IDENTIFICATION OF GENES DIFFERENTIALLY EXPRESSED IN RAT BRAIN DURING POSTNATAL DEVELOPMENT

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1 Introduction

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1.1.1 Axonal outgrowth in the developing CNS

How neurons extend their axons and, in particular, how this process is regulated is a fascinating problem as the correct growth of axons to their targets underlies the establishment of precise functional connections in the developing brain and is the basis for successful axonal regeneration and functional recovery after nerve injury. While the lengthening of a neurite occurs by extension of axially oriented bundles of microtubules in the axon, the direction and pattern of growth are directed by a highly specialized structure at the tip of each growing neurite, the growth cone. Both movements are coupled and thereby lead to a directional elongation of the axon. Growth cones resemble finger like structures that constitute the locomotory organelles of neurons and are extremely motile through a network of actin filaments at their leading edge. Time-lapse videomicroscopy elucidated growth cone dynamics and demonstrated that the growth cone advance is saltatory, with frequent advances and pauses, and that growth cones remain motile during pauses and make minute advances and retractions, accompanied by extensive cytoskeletal remodelling of the growing tip (reviewed by Mason and Erskine, 2000). Rapid extension was shown to occur along other fascicles of neurites when growth cones could follow the axons of pioneer neurons, pausing was predominant at choice points such as the CNS midline (reviewed by Mason and Erskine, 2000). In the course of axon extension, processes can fasciculate to form bundles with other axons or defasciculate to choose a separate pathway and they can branch.

For most CNS neurons of higher vertebrates, the elongation of a principal axon is confined to a few days or weeks during development (Chen et al., 1997; Dusart et al., 1997; reviewed by Ferretti et al., 2003; Li et al., 1995; Prang et al., 2001; Woodhams et al., 1993). The molecular processes required for axon growth might be inactivated after this period in order to stabilize existing connections (reviewed by Fawcett, 1992; Skene, 1989). The growth-associated protein GAP-43, for example, which is important for axonal outgrowth during development and regeneration, is highly expressed in growth cones of axon-extending neurons and is strongly downregulated in many brain regions after neuronal maturation. Recently, it was suggested that a target-derived retrogradely transported repressive factor might account for this decline in GAP-43 expression (Karimi-Abdolrezaee and Schreyer, 2002; Woolf et al., 1990). As a consequence, such a developmental inactivation or retention of growth-promoting factors might lead to a strong decline of axon growth and regeneration in mature neurons. Previous transplantation and co-culture experiments, which revealed a strong intrinsic growth state of embryonic neurons in a mature environment, support this hypothesis (reviewed by Caroni, 1997; Chen et al., 1997; Dusart et al., 1997; Li et al., 1995; Prang et al., 2001; Woodhams et al., 1993). A few proteins associated with axon growth during both, development and regeneration, have been identified in recent years, which belong to different classes of molecules with diverse functions, ranging from signal transduction components and transcription regulators to cell surface molecules and growth-related proteins (reviewed by Caroni, 1997; Skene, 1989). Examples are GAP-43, CAP-23 and cell adhesion molecules (CAMs) (reviewed by Skene, 1989; Caroni, 1997. See also section 1.1.4). As these neuronal proteins are strongly expressed in axon-extending neurons during development and are re-induced after nerve lesion, they might be part of an intrinsic molecular elongation program of a neuron. Extracellular matrix molecules are also able to promote or restrict axon growth and to modify the behaviour of growth cones in response to environmental cues. Laminin, for example, is growth promoting, whereas versican and certain tenascins impede axonal growth (Asher et al., 2002; Hopker et al., 1999; Schmalfeldt et al., 2000). Furthermore, cell-adhesion molecules of the Ig-superfamily are known to subserve several functions during developmental axon growth, for instance fasciculation, interaction with cell surface molecules and pathway selection (Dodd et al., 1988; reviewed by Rutishauser and Jessell, 1988) and they support regeneration of retinal ganglion cells (RGCs) in fish (Stuermer et al., 1992). Neurotrophic factors are another class of molecules that are required not only for neuronal survival but also for neuronal differentiation (reviewed by Bibel and Barde, 2000; Davies, 2000; Huang and Reichardt, 2001; McAllister, 2001). Distinct neurotrophins are secreted by target cells from which they are retrogradely transported to the outgrowing neurons *in vivo* (Ginty and Segal, 2002) and some neurotrophins are sufficient to induce neurite processes in PC12 cells *in vitro* (reviewed by Vaudry et al., 2002).

Axon pathfinding and guidance is one aspect of the differentiation program that occurs in postmitotic neurons and polarizes the cells. As an axon grows away from the neuronal cell body, it must choose among various substrates and recognize environmental cues in order to reach its specific target. It then must recognize and enter the target area, where extensive terminal arborisation occurs, and synapse with the appropriate cells. These processes depend on the interpretation of combinations of incoming signals by particular growth cones. Molecules outside of growth cones which influence the direction of axon outgrowth – the environmental cues – are relevant as well as molecules that are expressed in and on growing axons, which include receptors for guidance cues and elements of signal transduction pathways that mediate changes in growth cone behaviour (reviewed by Daston and Koester, 1996; Keynes and Cook, 1995; Schwab, 1996). Among the environmental cues that attract or repel axons are the netrins, which have been shown to function as midline chemoattractants (Kennedy et al., 1994; Serafini et al., 1994), members of the semaphorin or collapsin family that are important for the patterning of CNS projections by growth-repulsion and -attraction (Fan and Raper, 1995; Messersmith et al., 1995; Puschel et al., 1995), the ephrin-Eph receptor system, which is known to play a role in contact repulsion and retinotectal map formation, and the *Robo-Slit/Comm* system involved in midline guidance (reviewed by Cook et al., 1998; Holland et al., 1998; Valtorta and Leoni, 1999; Wilkinson, 2001; Yamamoto et al., 2002). Different mechanisms for growth cone guidance have been described: contact attraction and contact repulsion, mediated by short-range cues such as cell surface- and matrix-associated components, as well as chemoattraction and chemorepulsion, mediated by diffusible long-range cues (reviewed by Tessier-Lavigne and Goodman, 1996). Yet, some guidance molecules are not exclusively attractive or repulsive, but rather bifunctional, depending on multiple features, for instance the type of neuron, developmental age, presence of other guidance molecules acting on the same growth cone and modulations of signal transduction pathways used by guidance molecules (reviewed by Mueller, 1999; Shewan et al., 2002). Moreover, a single growth cone can respond to the same guidance cue in opposing ways depending on the level of intracellular signalling molecules such as calcium or cyclic nucleotides (reviewed by Mason and Erskine, 2000; Song et al., 1997). It is able to integrate the information of multiple guidance cues in a highly coordinated, hierarchical manner and to translate them into proper behavioural changes. Signalling mechanisms responsible for triggering axon extension have been identified in recent years and provided a link between guidance cues and the cytoskeletal organization of a growth cone. For instance, Rho-like GTPases, a family of small GTP-binding proteins including Cdc42, Rac and Rho are activated by axon guidance receptors and are highly expressed in certain brain regions during periods of neurite outgrowth. They function in the formation of growth cone filopodia and lamellipodia as well as in growth cone collapse by indirectly targeting the actomyosin system at the leading edge (reviewed by Mueller, 1999; Patel and Van Vactor, 2002; Yuan et al., 2003). The importance of so-called lipid rafts in growth cone signalling and migration has recently become evident. It was shown that two CAMs, L1 and N-cadherin, but not ß1-integrin, are regionally concentrated in specialized microdomains of the growth cone cell membrane and that disruption of these lipid rafts prevented migration of growth cones on L1 or N-cadherin substrates, yet growth cone motility was unaffected on a laminin substrate, to which ß1-integrin binds (Nakai and Kamiguchi, 2002). Moreover, it was demonstrated that the localization of distinct proteins to lipid rafts of the growth cone membrane is regionally regulated in a neuron and becomes altered during development, thereby regulating signal transduction through these cell surface molecules in a precise way (Nakai and Kamiguchi, 2002). In addition, calcium transients were shown to occur in growth cones *in vitro* and *in vivo* that determine the rate of growth cone advancement (Gomez and Spitzer, 1999; Gomez and Spitzer, 2000).

To conclude, molecules that were found to be associated with various aspects of axon growth such as axon extension, adhesion, attraction, repulsion, fasciculation and branching encompass neuronal proteins as well as extracellular molecules, associated with glial cells or the extracellular matrix, and diffusible factors. Thus, it seems that axon growth is not determined by a single molecule but rather is an interplay between multiple factors, which renders the control of a neuron's growth state very complex. Neuron-intrinsic molecules appear to likewise play a role in this process, as do environmental cues.

1.1.2 Axonal regeneration in the CNS

The response to injury and the potential for axonal regeneration in the CNS are highly variable and depend largely on the neuronal cell type affected, the developmental age, type of injury and length of the remaining myelinated axon segment. Moreover, regeneration of CNS neurons differs largely in different organisms. Whereas a good axonal regeneration with functional recovery is observed in the CNS of fish and amphibians (Stuermer et al., 1992) and in the PNS of higher vertebrates, the regenerative response in the CNS of birds and mammals is very poor (Kapfhammer, 1998). Mature CNS neurons of higher vertebrates are unable to regenerate their axons over long distances and to reconnect to their target cells. However, central neurons show some degree of plasticity called axonal sprouting, which occurs at cut axon stumps close to the lesion site but is different from long distance growth (Caroni, 1997). For instance, adult DRG neurons, which are bipolar neurons possessing a peripheral and a central axon branch, show extension of long axons in culture after peripheral axotomy but display a distinct growth mode characterized by less elongation and higher branching after central transection. The transition between distinct modes of axon growth depends on different patterns of gene expression that are partly triggered by the lesion-induced interruption of axonal transport (Smith and Skene, 1997). Despite the failure of lesioned CNS neurons to regenerate, a process called collateral sprouting occurs in uninjured neurons, which are located close to the denervated target cell and can form axon collaterals and sprout into the denervated region, thereby functionally compensating to some part for the nerve fibers lost by the lesion (reviewed by Kapfhammer, 1997). Yet, collateral sprouting and axonal regeneration are highly age-dependent processes which decrease considerably with developmental maturation of the CNS *in vitro* as well as *in vivo* (Chen et al., 2000; Dusart et al., 1997; reviewed by Kapfhammer, 1997; Li et al., 1995; Prang et al., 2001; Woodhams et al., 1993).

Another aspect of CNS regeneration is the induction of an extensive gliosis by reactive astrocytes and microglial cells at the lesion site, which can proliferate, migrate into the lesion site, phagocytose axonal debris, remove synapses and produce pro-inflammatory cytokines (reviewed by Turner et al., 1998). Glial activation results in the formation of a glial scar containing inhibitory extracellular matrix molecules such as chondroitin sulphate proteoglycans (CSPGs). Thus, the glial scar represents a physical and inhibitory barrier to axonal regrowth, which might contribute to the failure of regeneration (reviewed by Stichel and Muller, 1998). Yet, in microlesions that leave intact the glial environment, CNS axons still do not regenerate (Davies et al., 1996), indicating that multiple restricting mechanisms must exist. Although activated glial cells might be detrimental to the injured neuronal network by expression and secretion of pro-inflammatory and growth-impeding molecules, they also might be beneficial by expression and secretion of cell adhesion molecules, e.g. PSA-NCAM and N-cadherin, growth factors such as nerve growth factor (NGF), ciliary neurotrophic factor (CNTF) or fibroblast growth factor (FGF), and by influencing the composition of the extracellular matrix (Aubert et al., 1995; Rudge and Silver, 1990; reviewed by Turner et al., 1998). Furthermore, growth factors are secreted by many target cells and are retrogradely transported to the appropriate axon-extending neurons. Interruption of the axonal transport, e.g. by nerve transection, thus might contribute to the poor regenerative capability of many CNS neurons due to the lack of growth-promoting factors (DiStefano et al., 1992).

The success of axon regeneration in the adult mammalian brain depends on the presence of growth-permissive environmental conditions as well as specific properties of the affected neurons. Additional factors restricting the regenerative response of CNS neurons are, for example, the CNS environment, which displays unfavourable growth conditions. It has been shown that components of CNS myelin, such as the NI-35/250 antigens, later identified as the Nogo proteins (Chen et al., 2000; GrandPre et al., 2000; Prinjha et al., 2000), and the myelin-associated glycoprotein MAG (McKerracher et al., 1994), induce growth cone collapse and, by inhibiting neurite-growth, crucially account for the failure of axon regrowth and restriction of collateral sprouting after CNS lesions (Caroni and Schwab, 1988b; reviewed by Kapfhammer, 1997; Li et al., 1996; Schwab et al., 1993). Consistent with this is the situation in fish, where oligodendrocytes lack growth-impeding molecules and, instead, support axonal elongation of fish as well as rat retinal axons (Stuermer et al., 1992). Conversely, sprouting of lesioned axons in mammals is considerably enhanced after unilateral pyramidotomy and lesion of the dorsal root, respectively, in the absence of myelin (Schwegler et al., 1995; Vanek et al., 1998). Moreover, two chondroitin sulfate proteoglycans, brevican and versican, that are expressed on differentiated oligodendrocytes are additional components of CNS myelin that contribute to its nonpermissive substrate properties for axonal growth (Asher et al., 2002; Niederost et al., 1999; Schmalfeldt et al., 2000). The presence of several myelin-associated growth inhibitors that act independently might explain why in MAG-deficient mice axonal regeneration is not improved (reviewed by Bartsch, 1996; Bartsch et al., 1995). The capacity of CNS neurons for regeneration declines sharply with the appearance of mature oligodendrocytes and myelin. Accordingly, the growth potential is not impeded in young CNS tissue with immature oligodendrocytes that do not induce growth cone collapse (Schwab and Caroni, 1988). Thus, the regenerative potential of CNS neurons correlates inversely with the maturation of oligodendrocytes expressing myelin-associated growth inhibitors. Yet, the ability of retinal ganglion cells to extend neurites on optic nerves is lost before the optic nerve becomes myelinated (Ferretti et al., 2003; Shewan et al., 1993) and regeneration remains limited in regions where myelin is not a major component of the scar such as in CNS grey matter or in non-myelinated retina (Rudge and Silver, 1990), suggesting that lack of myelin proteins is not sufficient for the occurrence of regeneration. This points to the existence of further growth-determinants. Embryonic neurons are capable of extending fibers in the inhibitory environment of adult CNS tissue and even can, for some neuron types, grow longer axons than in the absence of myelin-associated molecules, indicating that a developmental change in the responsiveness of CNS neurons to myelin-associated growth inhibitors occurs (Bandtlow and Loschinger, 1997; Davies et al., 1993; Li and Raisman, 1993; Shen et al., 1998). Accordingly, after implantation into a CNS lesion site of an adult animal, embryonic striatal neuroblasts exhibit long fiber growth on non-permissive CNS tissue to reach their appropriate targets (Wictorin et al., 1990). These results demonstrated that the growth-inhibition by a non-permissive environment can be overcome by neurons which exhibit a strong intrinsic growth potential and might lack receptors for the inhibitory myelin-associated molecules and that a loss of intrinsic growth capacity occurs during development. Such an intrinsic axon elongation program might differ from a terminal sprouting and branching program which implies that a switch in gene expression might direct a distinct type of growth mode (Caroni, 1997). Recently, Goldberg et al. demonstrated that for retinal ganglion cells this switch could be induced by contact to amacrine cells, i.e. by a contact mediated signaling event at the dendrite of the neurons (Goldberg et al., 2002b). This result is consistent with the finding by Dusart et al., who described that the decrease in regenerative potential of Purkinje neurons correlates with the onset of synaptogenesis and dendritic remodelling of these cells (Dusart et al., 1997).

Intrinsic determinants that might influence the axon growth capability are, for example, the growth-associated proteins GAP-43 and CAP-23 (Laux et al., 2000; Skene and Willard, 1981a; Skene and Willard, 1981b), which are expressed abundantly at the growth cone surface during fiber outgrowth and synapse formation and which are downregulated in many brain regions during development when the growth capacity of CNS neurons strongly declines (Benowitz and Routtenberg, 1997; Caroni, 1997). Myelination of CNS axons and expression of the growth-associated protein GAP-43 in CNS neurons are complementary and are inversely correlated regarding the growth potential of these neurons: high levels of GAP-43 expression combined with light myelination promote neuronal plasticity, whereas weak expression of GAP-43 but dense myelination restrict plasticity (Kapfhammer and Schwab, 1994a; Kapfhammer and Schwab, 1994b). A further candidate molecule that determines the growth potential of CNS neurons is the anti-apoptotic protein bcl-2, which, independently of its function to promote cell survival, stimulated regeneration of severed retinal ganglion cell axons in culture and *in vivo* (Chen et al., 1997). Yet, this effect could not be reproduced in the same type of neurons and more recent studies pointed to a sole role of bcl-2 in promoting survival of injured RGCs (Chierzi et al., 1999; Goldberg et al., 2002a; Lodovichi et al., 2001). Another determinant, cAMP, was revealed by various groups to be implicated in signalling a neurons growth response. For instance, when supplied simultaneously with neurotrophic factors, physiological levels of electrical activity or cAMP promote neurotrophin responsiveness and dramatically potentiate axon outgrowth of RGCs (Goldberg et al., 2002a). Moreover, high levels of cAMP can overcome the inhibition of axonal growth by myelin components (Cai et al., 1999) and protein kinase A (PKA) and polyamines were shown to be downstream targets of this signalling pathway in DRG neurons (Cai et al., 2002).

Improvements of axonal regeneration:

Several approaches to encourage regeneration have focused on addressing individually the varying impediments to growth. As axonal growth is dually regulated by extrinsic and intrinsic determinants, experimental approaches that aimed at the improvement of CNS regeneration intended to both, rendering the inhibitory CNS environment growth-permissive for regenerating axons and stimulating the intrinsic growth properties of lesioned CNS neurons. As a result, regeneration of CNS axons was found to be improved under certain experimental conditions with limited functional recovery in some cases, but a regrowth of most fiber bundles with a complete re-innervation of the appropriate target was never achieved. Some approaches are outlined below:

Reduction of reactive gliosis or eliminating the glial scar and its inhibitory components, either by gliotoxic drugs or by collagenase digestion, results in improved regeneration of CNS nerve fibers (Gimenez y Ribotta et al., 1995; Hermanns et al., 2001; Stichel et al., 1999; reviewed by Stichel and Muller, 1998). Removal of glycosaminoglycan chains from chondroitin sulphate proteoglycans (CSPGs) attenuates CSPG inhibitory activity, thereby promoting regeneration of severed axons and leading to functional improvements in the CNS *in vitro* and *in vivo* (Bradbury et al., 2002; Moon et al., 2001; Zuo et al., 1998). A conditioning lesion (a prior injury of the peripheral branch of DRG neurons followed by a second axotomy of the central branch) stimulates the axonal growth program of DRG neurons as shown, for example by upregulation of GAP-43, and increases the sprouting response of their central branches into the denervated target area in the spinal cord (reviewed by Kapfhammer, 1997).

Neutralization or inhibition of myelin components with the monoclonal antibody IN-1 or antibodies specific to Nogo leads to a pronounced regeneration of lesioned spinal cord axons in the adult rat, which is accompanied by functional improvements such as locomotor recovery (Bregman et al., 1995; Caroni and Schwab, 1988a; Merkler et al., 2001; Schnell and Schwab, 1990; Tatagiba et al., 1997; Thallmair et al., 1998; Z'Graggen et al., 1998). This is consistent with a significantly enhanced axonal sprouting response after lesion of the corticospinal tract in a mature rat in which myelination was inhibited by neonatal X-irradiation (Vanek et al., 1998). Moreover, blocking of the Nogo-66 receptor, which is involved in Nogo-mediated growth inhibition, by application of a competitive receptor antagonist elicited a clear axon sprouting response of the lesioned corticospinal tract in the rat and improved functional recovery (GrandPre et al., 2002). In addition, pharmacological inactivation of the rho-GTPase allowed retinal neurons to extend axons on a myelin substrate, which under normal conditions is inhibitory, and promoted CNS regeneration of retinal neurons after optic nerve crush *in vivo* (Lehmann et al., 1999).

Transgenic overexpression of growth-associated molecules like GAP-43 and CAP-23 in CNS neurons enhances axonal sprouting and plastic changes at axon terminals and synapses and stimulates neurite outgrowth in culture (Aigner et al., 1995; reviewed by Benowitz and Routtenberg, 1997; Holtmaat et al., 1997). Upon co-expression of CAP-23 and GAP-43 axon regeneration of DRG neurons was further potentiated *in vitro* and *in vivo* (Bomze et al., 2001). Transgenic overexpression of the anti-apoptotic protein bcl-2 enhanced survival and improved regeneration of retinal ganglion cells (Chen et al., 1997). Application of neurotrophins, e.g. by implanting cells genetically engineered to produce and secrete various trophic molecules, enhances local axonal sprouting and, to some degree, axonal regrowth of adult lesioned CNS neurons (reviewed by Bregman et al., 2002; Broude et al., 1999; Eagle et al., 1995; Gage et al., 1990; Grill et al., 1997; Liu et al., 1999; Schnell et al., 1994; Woodhams and Atkinson, 1996). Transplantation of embryonic nervous tissue, dissociated Schwann cells, peripheral nervous tissue or olfactory ensheating cells, all of which are permissive for axonal growth, favours regeneration of some axonal types but is not sufficient to promote regenerative axonal outgrowth in every neuronal cell type. Enhancement of CNS axon regeneration was first shown by grafting peripheral nervous system tissue into the lesion site (David and Aguayo, 1981; Richardson et al., 1980). Some of the regenerating fibers were shown to form functional connections in their target regions (Cheng et al., 1996; Keirstead et al., 1989; Sauve et al., 1995; Thanos, 1992). Later, either suspensions of dissociated Schwann cells that were injected into the lesion site or transplantation of Schwann cell seeded guidance channels or application of a

Schwann cell conditioned medium have also been shown to promote axon growth of CNS neurons (Li and Raisman, 1994; Stichel et al., 1996; Xu et al., 1995). Injections of suspensions of olfactory ensheating cells (OECs) into a corticospinal tract lesion induced axon elongation of the cut axons over the lesion site into the host tissue and resulted in successful functional improvements (Keyvan-Fouladi et al., 2002; Li et al., 1997; reviewed by Raisman, 2001). Although the majority of such experiments was done in spinal cord, successful regeneration could also be demonstrated in other CNS systems. Olivocerebellar fibers, for example, regenerate well and enter their target region under such permissive conditions (reviewed by Rossi et al., 1995) and neurons of the thalamic reticular nucleus exhibit an established propensity to regenerate their axons along the graft which is accompanied by upregulation of c-Jun and GAP-43 (Vaudano et al., 1998). Moreover, Schwann cell grafts promoted the regeneration of hippocampal fibers to the mamillary body after transection of the postcommissural fornix in adult rats (Stichel et al., 1996). Axonal regeneration and functional recovery of adult lesioned CNS neurons was similarly enhanced by grafting embryonic CNS tissue into the lesion site in combination with supply of neurotrophins (Coumans et al., 2001; Tuszynski and Gage, 1995).

In summary, these experiments elucidate that axonal re-growth in the CNS is possible under certain conditions but they also clearly show that, despite the remarkable achievements in promoting regeneration in the mammalian CNS, the success is still incomplete. Furthermore, it becomes clear that regeneration depends on the interplay between environmental cues and intrinsic properties of axotomized neurons.

1.1.3 Axonal outgrowth and regeneration in the rat cerebellum and entorhinal cortex during neuronal development

Cerebellum:

The time course of neurogenesis and axon outgrowth is well described in developing rat cerebellum. Purkinje neurons are born between E14 and E15 in the ventricular neuroepithelium, migrate superficially from E17 to settle beneath the external granular layer (EGL) and form a distinct Purkinje cell layer (Altman and Bayer, 1985). From E18 on, Purkinje cells project to their targets, the deep cerebellar nuclei and vestibular nuclei, with which they form inhibitory GABAergic synapses (Eisenman et al., 1991). Birth and axon growth of other cerebellar neurons occurs later in development and neurogenesis of granule cells even extends to P21 (Altman and Bayer, 1978). Purkinje cells are among the most resistant neurons to axotomy and the most refractory to axonal regeneration (Dusart et al., 1997). Lesioned Purkinje cells of the adult rat or mouse survive axotomy but do not regenerate their axons, which appear as thickened processes with large terminal clubs at their ends (Dusart and Sotelo, 1994; Rossi et al., 1995). Following transection of rat Purkinje cell axons in organotypic slice cultures, Purkinje neurons survive before or after a critical period during the first postnatal week, the time of extensive synaptogenesis, but they only regenerate their axons if taken from fetal animals. When axotomized at P7 or later, rat Purkinje neurons fail to re-grow their axons (Dusart et al., 1997). Thus, Purkinje cell survival and regeneration are obviously age-dependent (Gianola and Rossi, 2001). Rescue of Purkinje neurons by inhibiting protein kinase C (PKC) does not enhance their regenerative capability (Ghoumari et al., 2002). Even after rendering the CNS environment permissive by transplanting peripheral nerve or fetal CNS tissue into the lesion site, Purkinje neurons from older developmental stages (e.g. P10) still did not regenerate (Dusart et al., 1997). When olivocerebellar and Purkinje cell axons from adult rodents are severed in the cerebellar white matter and become confronted with target-specific embryonic tissue or dissociated Schwann cell grafts, the former regenerate vigorously into the transplant, whereas the latter invariably fail to do so (reviewed by Rossi et al., 1997; Rossi et al., 1995). Similarly, in contrast to neurons of the thalamic reticular nucleus, deep cerebellar nuclei and inferior olive, the cell body reaction in response to axotomy with subsequent grafting of growth-permissive tissue is only weak in Purkinje neurons, which do not upregulate c-Jun or GAP-43, correlating with very poor regenerative capabilities (Vaudano et al., 1998; Zagrebelsky et al., 1998). After neutralization of myelin-associated growth inhibitors by IN-1 antibody application, Purkinje neurons exhibited an enhanced cell body response with upregulation of Jun-proteins, indicating that inhibitory myelin components are involved in the retrograde regulation of injury-associated gene expression (Zagrebelsky et al., 1998). Targeted overexpression of GAP-43 in cerebellar Purkinje cells induced axonal sprouting after axotomy but not long distance regeneration of the lesioned axons (Buffo et al., 1997). After lesion of the adult cerebellar cortex, subsets of Purkinje neurons strongly upregulated low-affinity nerve growth factor receptor p75 and showed a beaded varicose morphology with the appearance of recurrent axonal collaterals (Martinez-Murillo et al., 1993). These examples demonstrate that the extent of cell body response is correlated to the regenerative potential and emphasize that a permissive local environment is not sufficient for successful regeneration of Purkinje cell axons but that intrinsic properties of axotomized neurons are important determinants for axonal regrowth as well (reviewed by Rossi et al., 1997; Zagrebelsky et al., 1998).

Entorhinal cortex:

Pyramidal cells of layers II and III of the entorhinal cortex project their axons to the ipsi- and contralateral hippocampal formation to innervate the granule cells of the dentate gyrus and the pyramidal cells of the hippocampal CA-region. The perforant pathway terminates laminar-specific in the outer molecular layer of the fascia dentata and in the stratum lacunosum moleculare of the hippocampus proper where it forms excitatory glutamatergic synapses with the pyramidal neurons of the cornu ammonis regions CA1 to CA3 (Frotscher, 1991). During development, axons of the entorhinal-hippocampal projection are fist observed at E17 in the rat, yet the majority of fibers develop around birth and begin to form synapses from days 3-5 postnatally (Ceranik et al., 2000; reviewed by Turner et al., 1998). It was shown that Cajal-Retzius cells, which seem to be required for layer-specific termination of entorhinal afferents (Super et al., 1998), form a pioneer projection from the hippocampus to the entorhinal cortex to provide a template for entorhinal axons that later grow out in the opposite direction (Ceranik et al., 2000). In addition, hitherto unidentified attractive, membrane-associated molecules, restricted to the proper target region of entorhinal fibers at a distinct developmental stage, seem to influence the ingrowth of these hippocampal afferents (Skutella et al., 1999). Several molecules known to function in axon guidance, for example semaphorins and ephrins were shown to be involved in establishing the entorhinal-hippocampal connection (reviewed by Skutella and Nitsch, 2001; Stein et al., 1999; Steup et al., 1999).

After lesion of the perforant path in an organotypic slice culture model, young entorhinal fibers were shown to re-grow their axons and to terminate in a layer-specific way, just as *in vivo* (Frotscher et al., 1997). Furthermore, commissural fibers from the contralateral hippocampus exhibit a reactive sprouting response after lesion, which is also layer-specific and leads to new synapses on the denervated dendrites (reviewed by Bechmann and Nitsch, 2000; Savaskan and Nitsch, 2001). The culture model, in which entorhinal cortex slices are co-cultured with hippocampal slices, revealed that entorhinal fibers re-innervate the hippocampus only if perforant path lesions are made before 2-3 weeks in culture, equivalent to a postnatal age of 11-18 days, and that this developmental decline in regenerative capability is due to maturation of the entorhinal neurons and not that of the hippocampal target (Li et al., 1995; Prang et al., 2001; Woodhams et al., 1993). Treatment with trophic factors after perforant path lesion, e.g. by addition of a Schwann cell conditioned medium or fibroblast growth factor, can ameliorate the decline in regenerative ability of entorhinal pyramidal neurons with increasing developmental age (Woodhams and Atkinson, 1996). Moreover, Prang et al. showed an improvement of regeneration after application of the neurotrophins GDNF and NT-4 or by inhibiting intracellular signalling molecules such as G-proteins or protein kinase C (Prang et al., 2001).

1.1.4 Axon growth-associated molecules

As axon elongation is confined to a few days or weeks during development and some growth-associated genes are downregulated in neurons after this period, it was suggested that maturation of a neuron is tightly linked to a repression of growth-related properties after completion of neuronal differentiation. Accordingly, the expression of some of the genes involved in axon growth might be transient during development and might be re-induced during successful axon regeneration (reviewed by Skene, 1989). Consequently, it was searched for genes whose expression is correlated consistently with periods of axon growth during both development and regeneration and whose expression is repressed as neurons mature (reviewed by Skene, 1989). In the past, a few proteins were identified that are very rich in axons or growth cones during fiber elongation and are tightly correlated with a neuronal “growth state”. These include the growth-associated proteins GAP-43 and CAP-23, actin and tubulin isoforms, the c-*src* tyrosine kinase and rho-GTPases, the neural cell adhesion molecule NCAM and its poly-sialylated form PSA-NCAM and the microtubule-associated proteins tau and MAP5 (Caroni, 1997; reviewed by Ferretti et al., 2003; Patel and Van Vactor, 2002; Skene, 1989; Tucker et al., 1989). The number of gene families and molecules identified increases continuously and further proteins had been shown to be involved in different aspects of neurite outgrowth and regeneration, e.g. neurotrophic factors, integrins, extracellular laminins, transcription factors of the immediate early gene (IEG) family and chemorepulsive proteins (Caroni, 1997; Goldberg et al., 2002a; Holtmaat et al., 1998).

Various groups identified differentially expressed genes by comparing the expression patterns of regenerating with non-regenerating systems on the one hand and gene expression in neurons before and after injury on the other hand. Altered expression was examined, for example, in neurons of dorsal root ganglia after sciatic nerve lesion (Mladinic and Wintzer, 2002; Newton et al., 2000; Xiao et al., 2002). Nevertheless, these approaches are problematic, because they also target genes that are not involved in long distance axon growth but rather are associated with general inflammatory reactions of the lesioned neurons. A few proteins were identified that are linked to different aspects of axon growth. Yet, no major regulators have been described so far that could mediate an intrinsic switch from a neuronal growth state to a “non-growth” state which allows stabilization of present axonal connections but restricts further axon growth. An important step towards understanding the molecular mechanisms underlying axon growth therefore will be to identify regulatory signals that, by controlling the expression of growth-associated genes, might determine a neuron’s propensity for axon growth. Possible regulatory signals are, for example, transcription factors. Hence, this protein class is outlined in the next chapter, as transcription factors were the main focus of the first approach of the present studies.

1.2 Transcription factors

Transcription factors are sequence-specific DNA binding proteins, which mediate changes in gene expression patterns in a cell in response to a specific challenge. They can act both as activators or repressors of gene transcription and exert this function by binding to discrete DNA motifs within and flanking a gene in a region of active chromatin, thereby determining the activity of the gene’s promotor. Some of the DNA motifs are conserved and share common consensus DNA sequences which are recognized by transcription factor families, which themselves are often expressed in a tissue-specific, developmental or stimulus-specific manner. Members of one protein family share a conserved DNA-binding region, which allows binding to a specific consensus DNA motif. Transcription factors can enhance their transactivation potency by operating in a combinatorial fashion, forming hetero- or homodimers or interacting with other (multimeric) transcription factor complexes, which are bound to DNA nearby. Thus, various affinities for specific consensus sequences are obtained and a large diversity in the regulation of gene expression is guaranteed (Sockanathan, 2003; Struhl, 1991). Transcription factors are composed of different domains that serve distinct functions. In general, they possess a DNA-binding domain for binding to specific promotor or enhancer DNA regions of a target gene, a transactivation domain for interaction with the basal transactivation complex and sometimes an additional dimerization domain in order to form homo-or heterodimers with other transcription factors. Transcription factors often act sequentially in that they regulate the expression of other transcription factors, which in turn regulate the expression of downstream effector genes.

1.2.1 Transcription factor classes

Depending on whether transcription factors are induced by cellular stimulation or are present in quiescent cells in the absence of any external stimulus, one can discriminate between inducible transcription factors, for instance the Jun, Fos and Krox proteins which are encoded by immediate-early genes, and constitutive transcription factors, e.g. the basic leucine zipper proteins CREB or ATF-1, which are already bound to DNA and are activated by phosphorylation (reviewed in Herdegen and Leah, 1998). Another classification groups transcription factors into families of related proteins which share peptide domains involved in either specific DNA sequence recognition or dimerization with related proteins (He and Rosenfeld, 1991; Latchman, 1993; Struhl, 1991). Being characterized by a common DNA binding domain, members of a specific family of transcription factors hence recognize similar DNA motifs in promotor and enhancer regions of downstream target genes. In the following section, six examples for such transcription factor families are described in more detail as they were of interest for the studies reported in the first results section of this thesis.

POU-domain proteins: The name POU was derived from four transcription factors which were first recognized to make up this protein family. These are the mammalian factors Pit1 and the octamer binding proteins Oct1 and Oct2, furthermore the protein Unc86 from C.elegans (Herr et al., 1988). Within the POU family, transcription factors are further classified in class I to class V proteins according to the similarity of their common POU domain, which constitutes the bipartite DNA binding domain of these proteins (He et al., 1989; Rosenfeld, 1991). The POU domain consists of a 70-80-amino acid POU specific domain and a 60-amino acid POU homeodomain related to that found in homeobox (HOX) proteins, which are depicted below. Both domains are linked by a variable and poorly conserved spacer region (see also *fig.3-2* in chapter 3). POU family members recognize a defined DNA sequence motif, known as the octamer element (He et al., 1989; Herr et al., 1988; Rosenfeld, 1991; Verrijzer and Van der Vliet, 1993; Wegner et al., 1993).

Basic helix-loop-helix (bHLH) proteins share a common structural motif of about 60 amino acids, which is characterized by two α-helices, separated by a loop. The helices mediate dimerization with homologous or heterologous bHLH proteins. The adjacent basic region fits in the major groove of the DNA and is required for DNA binding to the E-box hexamer CANNTG (Murre et al., 1989a; Murre et al., 1989b). The nucleotides of this DNA recognition motif vary in different subfamilies of bHLH transcription factors, which are grouped on the basis of closer sequence similarities in the bHLH domain. Subfamilies of bHLH proteins are, for example, the atonal family, including the *Math* proteins, the olig family with the *olig* factors, the achaete-scute family, which comprises the *Mash* proteins, the NeuroD family with *NeuroD-* and *Math* factors and the neurogenin family, which contains the *neurogenin (Ngn)* proteins (reviewed by Bertrand et al., 2002).

Zinc finger proteins consist of a motif in which either four cysteine residues or two cysteine and two histidine ligands coordinate a zinc ion (C_2-C_2 type or C_2-H_2 type). In most proteins, this domain is present in multiple copies as regular tandem arrays with invariant cysteine and histidine residues in each repeat. Every single motif hence forms an independently folded structural domain organized around a zinc ion, which is essential for correct folding. The polypeptide fold consists of a helix and two ß-strands that are arranged in a hairpin structure (Lee et al., 1989) see also *fig.3-4* in chapter 3). The zinc finger domain mediates specific DNA recognition and -binding of transcription factors and is also used in protein-protein interactions (Berg, 1990; reviewed by Bertrand et al., 2002; Evans and Hollenberg, 1988; Miller et al., 1985). The vast majority of zinc finger proteins can be classified as Krüppel-like on the basis of a shared, highly conserved sequence stretch, the H/C-link, which connects consecutive finger repeats (Schuh et al., 1986).

Homeobox transcription factors were originally proposed to constitute a multigene family in Drosophila melanogaster by the group of Walter Gehring in the biocenter in Basel who reported that these homeotic genes reside in distinct chromosomal gene complexes (the Hox-clusters) and function in the specification of body segments during embryonic development (Kappen et al., 1993; McGinnis et al., 1984; Moretti et al., 1994; reviewed by Scott et al., 1989). Genes that code for members of the homeodomain family of transcription factors contain a common sequence of about 180 bp (the homeobox) that encodes a DNA-binding protein sequence known as the homeodomain. The structure of the 61-amino acid homeodomain is constituted by three α-helices (helix-turn-helix) and is highly conserved across species (reviewed by Kappen and Ruddle, 1993). Nevertheless, apart from this domain, Hox proteins are highly diverged and can be grouped into subclasses upon closer sequence similarity (McGinnis et al., 1984; Scott et al., 1989).

ETS-domain transcription factors share a highly homologous 80-90-amino acid DNA-binding domain, the ETS domain, which was first discovered in oncogenes cloned from retroviruses (Karim et al., 1990). In addition to directing specific protein-DNA interactions, the ETS domain also mediates protein-protein interactions (Nye et al., 1992; Sharrocks, 2001; reviewed by Wasylyk et al., 1993). Outside the conserved DNA-binding domain, ETS factors reveal a pronounced divergence and can be grouped into subclasses based on additional homologous regions unique for particular members of the ETS family (Janknecht and Nordheim, 1993; Wasylyk et al., 1993)

Forkhead proteins share a winged helix DNA-binding domain of about 100 amino acids, which folds into a variant of the helix-turn-helix motif and is made up of three α-helices and two characteristic large loops, or "wings" (Kaestner et al., 2000; Lim et al., 1997). In 1998, a standardized nomenclature for all chordate winged helix / forkhead transcription factors was introduced and Fox (Forkhead box) was adopted as the unified symbol for these proteins (Kaestner et al., 2000). Apart from the conserved DNA-binding domain, Fox proteins are highly divergent and can be sub-classified in 15 subclasses (Kaestner et al., 2000).

1.2.2 Transcription factors are key regulators during nervous system development and regeneration

Transcription factors are differentially expressed in a developmental, tissue-specific or stimulus-inducible manner. Descriptions of discrete expression patterns of transcription factors during nervous system development and analyses of mutant phenotypes reveal the regulatory role of region- and cell-type specific transcription factors in morphogenesis and differentiation of the vertebrate nervous system (reviewed by Bang and Goulding, 1996). The neurogenic bHLH transcription factor Nex1/Math-2, for example, displays its peak expression during brain development when neurite outgrowth and synaptogenesis are highly active and is a key activator of the growth-associated GAP-43 gene (Uittenbogaard et al., 2003). Knockout experiments, in which the expression of distinct transcription factors was disrupted, have pointed out important roles transcription factors play during nervous system development. CREB null mice, for example, display defects in axonal projections within two major brain commissures (Lonze and Ginty, 2002), deletion of ATF-2 (=CREB2) results in a disturbed organization of the cerebellum with reduced numbers of Purkinje cells (reviewed by Herdegen and Leah, 1998). Disruption of the zinc finger protein Krox-20 leads to the loss of two hindbrain rhombomeres and some motoneurons and is accompanied by insufficient myelination and misrouting of growing axons (Herdegen and Leah, 1998). Brn-3a knockout mice exhibit enhanced neuronal cell death and severe differentiation and migration defects in sensory and motor neurons (McEvilly et al., 1996; Xiang et al., 1996). Moreover, the Emx2 homeodomain protein, whose function was likewise revealed by deletion of the gene, is essential for terminal differentiation of granular cells of the dentate gyrus and plays a role in neuronal migration and formation of proper axonal connections in hippocampal, olfactory as well as thalamo-cortical circuits (Mallamaci et al., 2000; Savaskan et al., 2002; Yoshida et al., 1997).

Many transcription factors are differentially regulated in a temporal and spatial fashion in the brain during postnatal development, which emphasizes their role during CNS development. The fos-proteins, for example, are downregulated in brain during development (Alcantara and Greenough, 1993), as also are two short isoforms of the brain basic helix-loop-helix factor 1 (Kawakami et al., 1996). As dividing progenitor cells differentiate into distinct phenotypes, there is a co-ordinated activation and repression of a variety of genes, which are under the control of complex regulatory networks of transcription factors. These are first induced by concentration gradients of diffusible signals such as sonic hedgehog (shh) or bone morphogenetic protein (bmp) which progenitor cells are able to translate into expression of a specific set of transcription factors (Gurdon and Bourillot, 2001). By acting in a sequential fashion and regulating downstream target genes, transcription factors expressed in progenitor cells play an important role in determining the cell’s fate (reviewed by Jurata et al., 2000; Lee and Pfaff, 2001).

Similarly, a set of transcription factors in postmitotic neurons is responsible for determination of the neurons mature phenotype by regulating the expression of terminal differentiation genes (reviewed by Lee and Pfaff, 2001). The POU-domain transcription factor unc-86, for example, is necessary for the synthesis and packaging of serotonin and plays a role in neurite outgrowth in specific serotonergic neurons in C.elegans (Sze et al., 2002). The ETS-transcription factor Pet-1 is an additional marker of central serotonergic neurons and activates the transcription of several genes, whose expression is characteristic of the serotonergic neuronal phenotype (Hendricks et al., 1999).

In C.elegans, a sequential action of transcription factors is found in motor neurons: they express a Hox transcription factor that regulates the expression of two further transcription factors of the POU- and zinc finger family, which in turn control neuronal differentiation processes, e.g. neuronal migration (Baum et al., 1999). Likewise, in the developing spinal cord and telencephalon of vertebrates, progenitor cells residing in defined progenitor domains give rise to different types of cells. Progenitor domains are characterized by differential expression of unique combinations of homeodomain transcription factors such as the *Pax-, Emx- or LIM homeobox (Lhx)* proteins and bHLH transcription factors, e.g. *Olig2*, *Mash1* and the proneural *neurogenin (Ngn)* proteins (reviewed by Lee and Pfaff, 2001; O'Leary and Nakagawa, 2002; Schuurmans and Guillemot, 2002; Shirasaki and Pfaff, 2002; Tsuchida et al., 1994). If this patterned expression of transcription factors is perturbed, progenitor cells give rise to different types of cells and exhibit changes in their migration pattern and neuronal axon pathfinding, which reveals, that these transcription factors are essential for arealization, cell fate determination, neurogenesis and neuronal subtype specification with the ability to select specific axon pathways (Nakagawa and O'Leary, 2001; reviewed by Schuurmans and Guillemot, 2002; Sharma et al., 2000; Sharma et al., 1998). Distinct classes of neurons thus can be recognized based on their expression profiles of transcription factors (Ericson et al., 1997; Moran-Rivard et al., 2001; Sharma et al., 1998; Tanabe et al., 1998). In the same way, neurogenesis and neuron subtype specification in the developing retina are regulated by a highly defined expression of proneural bHLH proteins (reviewed by Vetter and Brown, 2001).

Furthermore, each developing neuron has been genetically programmed to be able to sense its unique pathway-specific guidance cues in order to reach its appropriate target regions (reviewed by Eisen, 1994). This intrinsic capacity is also conveyed by the expression of certain transcription factors (reviewed by Pfaff and Kintner, 1998). In Drosophila interneurons, the gene *apterous,* which codes for a LIM-homeodomain protein, has been shown to control pathway selection and axon fasciculation (Lundgren et al., 1995). The zebrafish homeobox gene *Noi* is required for commissural axon pathway formation across the midline of the diencephalon and might exert this function by regulating the expression of axon pathfinding molecules such as the netrins (Macdonald et al., 1997). In vertebrates, the *Vax1* homeodomain transcription factor is expressed by midline glial cells of the CNS and is implicated in commissural axon crossing at the midline by activating the expression of guidance cues such as netrin or the receptor protein-tyrosine kinases EphB2 and EphB3 (reviewed by Lemke, 2001). *Pax-6* is required for proper pathfinding of the postoptic commissural tract (Mastick et al., 1997) and certain LIM-homeodomain proteins are important for axonal pathfinding of cranial motor nerves during development of the brainstem (Varela-Echavarria et al., 1996). Two homeodomain proteins *En-1* and *En-2*, homologues of the Drosophila segment polarity gene engrailed, as well as two forkhead transcription factors are involved in the establishment of a topographical retinotectal map in the developing chick (Itasaki and Nakamura, 1996; Yuasa et al., 1996). The ETS factors ER81 and PEA3 control establishment of functional sensory-motor circuitry in the developing spinal cord (Arber et al., 2000; Lin et al., 1998).

Many transcription factors are induced in response to neurotrophins such as NGF or BDNF, for example PAX family members (Kioussi and Gruss, 1994) and CREB (reviewed by West et al., 2002). The **e**arly **g**rowth **r**esponse gene, Egr1, is also induced in PC12 cells upon stimulation with NGF, which elicits differentiation of these cells into neuron-like cells (Milbrandt, 1987; Sheng and Greenberg, 1990). Recently, a leucine zipper transcription factor was identified, which is induced following NGF stimulation and regulates neurite outgrowth of immature telencephalic neurons (Torocsik et al., 2002).

Evidence has been attained that transcription factors are also involved in the cellular response to nervous system injuries as some of them are induced after axonal lesions, for instance the transcriptional regulators that belong to the group of immediate early genes (IEGs). c-Jun, as one example, is strongly increased in dorsal root ganglion cells and motor neurons after sciatic nerve lesion (Jenkins and Hunt, 1991). In rat retinal ganglion cells, a significant increase of Jun and KROX proteins and decrease of the CREB transcription factor occur transiently after optic nerve lesion and parallel axonal sprouting (Herdegen et al., 1993). In addition, Robinson et al. demonstrated that c-Jun is upregulated in regenerating but not in non-regenerating retinal ganglion cells after transection of the optic nerve with and without an implanted peripheral nerve graft, respectively (Robinson, 1995). c-Jun and JunB are strongly induced in facial motor neurons after peripheral transection of the facial nerve (Haas et al., 1993) and in medial septal neurons after axotomy of the septohippocampal projection (Haas et al., 1996). Jun proteins and, to a lesser extent, Krox-24, are also induced after transection of the rat medial forebrain bundle in the corresponding affected neurons, most likely due to an interruption of the axonal transport (Leah et al., 1993). The limited regeneration of CNS axons into peripheral nerve grafts can be correlated with the rapid loss of c-Jun expression in Schwann cells following exposure to the CNS environment (Vaudano et al., 1996). Activating transcription factor 3 (ATF3), which can form heterodimers with c-Jun, is induced after peripheral nerve axotomy in concert with c-Jun in sensory DRG neurons and motoneurons of the spinal cord (Tsujino et al., 2000). Nevertheless, despite the relationship between the expression of injury-induced genes, the so-called cell body response, and the regenerative potential of lesioned neurons, the precise role of such genes in axon growth is still unclear, if not controversial, because some of them can also induce cell death (reviewed by Herdegen et al., 1997; Vaudano et al., 1998). Moreover, c-Jun induction after axotomy is age-independent in medial septal neurons and occurs in young, regenerating as well as mature, growth-resistant septohippocampal neurons (Haas et al., 1998). It was demonstrated that the elevation of IEG products including c-Jun, which occurs in response to convulsant stimuli, is not obligatory for reactive mossy fiber sprouting in the hippocampus (Nahm and Noebels, 1998). Although the role and regulation of c-Jun in axotomized neurons is not yet clear, some other molecules involved in the regenerative response have been identified that might be regulated by c-Jun. The growth-associated molecule GAP-43, for example, is co-expressed in parallel with c-Jun in regenerating neurons after nerve transection (Caroni, 1997; Haas and Frotscher, 1998; reviewed by Herdegen et al., 1997).

Other transcription factors were also shown to be implicated in lesion-induced processes. The class III POU-domain transcription factor Brn-3a, for example, which plays a role in the survival of sensory neuronal populations during development, can enhance neuronal survival after peripheral nerve injury by inducing the expression of anti-apoptotic proteins, e.g. bcl-x (L) (Smith et al., 2001). The POU-domain transcription factor Oct-6 (also known as Scip or Tst-1) is a major regulator of Schwann cell differentiation and myelination during peripheral nerve development and also during nerve regeneration (Mandemakers et al., 2000), is re-expressed after injury and can accelerate axonal regeneration in transgenic mice (Gondre et al., 1998; Scherer et al., 1994). Oct-2 expression is transiently increased after peripheral axotomy of sensory neurons and therefore might also be important for regeneration (Begbie et al., 1996). In tailed amphibia that are capable of regenerating spinal cord axons after nerve transection or tail amputation, the *Sox1* homeobox transcription factor is upregulated following lesion and parallels spinal cord regeneration (Ferretti et al., 2003).

In summary, transcription factors are highly regulated during nervous system development and after nerve injuries and are involved in various aspects of neuronal development, e.g. the generation of appropriate neuronal cell types, establishment of specific neuronal connections and determination of soma positions, axonal projection patterns, dendritic morphologies, expression of specific ion channels, neurotransmitter receptors and appropriate neurotransmitters (Edlund and Jessell, 1999; reviewed by Jessell, 2000; Shirasaki and Pfaff, 2002). After nervous system lesions, some transcription factors are differentially regulated and changes in expression patterns could be correlated with regenerative processes in a few cases, although precise functions of transcription factors after lesions remain to be elucidated. Yet, the knowledge about gene regulation after nervous system lesions is still quite poor. Altogether, transcription factors constitute an important molecule class with crucial regulatory functions during nervous system development that are essential for the proper functioning of the mature brain and might be re-activated after nerve injuries in order to trigger regeneration.

1.3 Aim of the present studies

1.3.1 Identification of transcription factors highly expressed in CNS neurons during establishment of axonal connections

As not much is known about the signals that control and define a neuron’s growth state, the major interest of the studies presented here focused on the regulation of axonal outgrowth of CNS neurons during neuronal development and differentiation. In order to elucidate the molecular mechanisms that underlie developmental fiber outgrowth, the studies aimed at the identification of molecules, which are highly expressed during axonal outgrowth and differentiation and thus might be potential regulators of the neuronal growth state. Two brain regions, the cerebellum and entorhinal cortex, were previously examined in slice culture models and their axon growth behaviour is well described during neuronal maturation and after fiber transection *in vivo* and *in vitro*. The studies revealed the time span of massive axonal outgrowth of cerebellar Purkinje neurons and entorhinal pyramidal cells, therefore these two CNS regions were chosen for examination of the gene expression patterns of axon-extending CNS neurons during neuronal differentiation. In Purkinje neurons, maximal axonal outgrowth occurs from late embryonic development until the first postnatal days, in entorhinal pyramidal neurons, growth-competence is high at birth and in the first postnatal week. At later time points of development, the potential for axon growth decreases strongly until it is completely lost. Based on these studies, a first approach was set up, in which it was searched for molecules involved in regulating axonal outgrowth in rat cerebellum at E18 and entorhinal cortex at P0.

The search focused on a certain group of molecules, the transcription factors, as they are potential regulators of many downstream target genes and control several processes during neuronal development. Yet, very little is known about transcription factor expression during axonal outgrowth of cerebellar Purkinje cells or pyramidal cells of the entorhinal cortex. As the expression of axonal growth-associated genes might be modulated in response to changes in the complement of regulatory proteins present in a cell, it is of great interest to identify such molecules, e.g. transcription factors that might function as critical regulators of different aspects of axon growth. Assuming that regeneration-related changes in gene transcription in CNS neurons might reflect expression programs during the period of axonal outgrowth during development, transcription factors might have important regulatory functions during both, neuronal development as well as axonal regeneration. The aim of the first approach of the present studies was, therefore, to identify members of various transcription factor classes in rat cerebellum at E18 and entorhinal cortex at P0, the time of extensive axon outgrowth, by degenerative PCR. This method is based on the fact that transcription factors of a certain family share a highly conserved DNA-binding domain, which allows the identification of new family members by PCR using degenerate oligonucleotides that are specific for the most conserved region. The conserved DNA-binding domains of the POU-, homeobox- (Hox), basic helix loop helix- (bHLH), zinc finger-, forkhead- and ETS- transcription factor families are well described and their consensus sequences were therefore taken as a basis for the generation of degenerate oligonucleotides. In the past, transcription factors of various transcription factor families were cloned by the method of degenerate PCR from various tissues and these studies were helpful for the design of degenerate PCR primer sequences (Agata et al., 1998; Chuaqui et al., 1997; Dovat et al., 1998; Furukawa et al., 1996; Gorski et al., 1994; POU: He et al., 1989; Hromas et al., 1993; Jehn et al., 1994; FKH: King and Moore, 1994; ETS: Lopez et al., 1994; Mesa et al., 1996; HOX:Moretti et al., 1994; Murtha et al., 1991; Zinc finger: Pellegrino and Berg, 1991; bHLH: Peyton et al., 1996; Singh et al., 1991; Walters et al., 1997).

In summary, applying PCR with degenerate primers for various transcription factor families onto cDNA from rat cerebellum at E18 and rat entorhinal cortex from P0 should allow the isolation and identification of transcription factors from two CNS regions at the time when massive axonal outgrowth occurs.

For several reasons, which are outlined in section 3.1.5, this approach turned out not to be promising. Another approach was therefore set up, which was methodically different but followed a similar goal.

1.3.2 Identification of developmentally regulated genes by Suppression Subtractive Hybridisation (SSH)

For the second approach, a more general strategy was used. So far, the main concern was to identify molecules, which are highly expressed during developmental fiber outgrowth in the CNS and therefore might regulate the intrinsic growth state of a developing neuron. As embryonic CNS neurons are well capable of elongating and regenerating their axons but loose this ability during maturation, growth-associated molecules possibly become downregulated in later stages of development after the completion of axon tract formation. This question was taken into consideration for planning the next experimental procedures. Like the differential display method or microarray analyses, the technique of suppression subtractive hybridisation allows the comparison of two distinct mRNA-populations and the identification of genes primarily expressed in one of both populations. By applying SSH, genes that are equally expressed in any two populations are subtracted and thereby sorted out from both groups. Only those genes, which are principally expressed in just one of the two populations, are enriched and are available for further analysis. SSH thus seemed to be a valuable tool to examine developmental changes in gene expression patterns of CNS neurons.

In order to identify genes, which exhibit an abundant expression in developing, axon-extending CNS neurons but which are only weakly expressed in mature neurons that have completed fiber growth and lack regenerative long-distance growth, the second approach of the present studies aimed at the identification of genes, which are differentially expressed in Purkinje neurons and entorhinal pyramidal cells, respectively, during neuronal maturation. As pointed out in 1.1.3, previous experiments with slice cultures from cerebellum and entorhinal cortex had extensively examined the growth competence of Purkinje neurons and entorhinal pyramidal cells and their dependency on maturation of the respective neurons. The decrease in regenerative capability was shown to occur after the first postnatal week around P7 in cerebellar Purkinje cells and from about P10 in entorhinal pyramidal neurons. These studies thus served as a basis to determine the time points for subtraction approaches in rat cerebellum and entorhinal cortex, in which developmental changes of gene expression patterns were examined. Accordingly, P0 and P10 were defined as the developmental stages to be compared in entorhinal cortex, with P0 representing the age of growth-capability and P10 the stage of growth-incapability. For comparison of expression patterns during the change of growth-competence in Purkinje neurons, developmental stages E18 and P35 were chosen. A time point later than P7 was preferred for examination of growth-incapable cerebellar neurons as other cerebellar neurons, e.g. the granule cells, arise much later during development and are still born in the third week after birth. It was assumed that at P35 all axonal fiber bundles should have established in the rat cerebellum. Thus, in the cerebellar approach, transcripts present at P35 were planned to be subtracted from transcripts present at E18, thereby identifying genes which are predominantly expressed in the late embryonic stage, when massive axon outgrowth of Purkinje neurons occurs *in vivo* and when regenerative growth of Purkinje cell axons over long distances is possible *in vitro*. Similarly, in order to compare the transcriptional activity of axon-extending entorhinal cortex neurons with mature, growth-incapable EC-pyramidal neurons, transcripts from rat entorhinal cortex at P10 were planned to be subtracted from transcripts from entorhinal cortex at P0, resulting in the enrichment of genes which are predominantly expressed around birth when perforant path fibers grow towards their target, the granule cells in the dentate gyrus.

In brief, both subtraction approaches with maturing Purkinje cells and entorhinal pyramidal cells, respectively, should result in the identification of genes which are highly expressed during axonal outgrowth of these neurons in the rat brain but which are downregulated thereafter and are, if at all, only weakly expressed in the adult CNS when no axon growth over long distances occurs anymore.

2 Materials and Methods

Buffers and solutions used for the following protocols are described separately in section 2.15.

2.1 Polymerase chain reaction (PCR)

PCR was performed according to a standard protocol, unless other cycling-conditions are delineated in the text.

Standard-PCR:	initial denaturation step:	94°C – 2 min.	
	denaturation:	94°C – 45 sec.	30x
	primer-annealing:	55°C – 30 sec.	
	elongation:	68°C – 45sec.	
	final elongation step:	68°C – 7 min.	

Annealing and elongation temperatures as well as duration of cycling steps and number of cycles were adapted to the applied oligonucleotide primers (see 2.13.5), DNA-polymerases and DNA-templates. Elongation with the Taq- or PWO- DNA-polymerase was performed at 72°C. A hot start was performed by first heating the samples for 8 min. at 95°C and then adding the polymerase directly to each tube. Amplification with the Advantage 2 Polymerase Mix, which includes a hot start, was performed at 68°C. Any difference to the standard PCR is indicated in the accordant text part in the results section (chapter 3).

2.2 Purification, precipitation and quantification of DNA

DNA was purified with the PCR-Purification Kit or Gel-Extraction Kit from Qiagen according to the manufacturer's instructions. Precipitation was carried out with sodium acetate and ethanol following the standard protocols from (Sambrook and Russell, 2001), section A8.12, and DNA was then quantified by measuring the optical density in a UV-spectrophotometer at a wavelength of 260 nm.

2.3 Fluorescent DNA sequencing

Capillary sequencing of cloned DNA was performed with the ABI PRISM 310 genetic analyser using the ABI PRISM BigDye Terminators Cycle Sequencing Ready Reaction Kit. The DNA sample to be sequenced was amplified by PCR with the Terminator Ready Reaction Mix. The amplification product was then precipitated and the pellet resuspended in 17 µl Template Suppression Reagent and applied for capillary sequencing after denaturation at 95°C for 5 min. Sequence results were analysed with the program Chromas, version 2.22, Technelysium (free trial at http://www.technelysium.com.au/chromas.html).

2.4 Digestion of DNA with restriction endonucleases

Restriction digestion of DNA-fragments and plasmid vectors was applied for subsequent cloning or linearisation of circular plasmid DNA for further riboprobe-synthesis. 1µg of DNA was digested with 5-10 units of enzyme for 2 hours at the indicated temperature. The enzyme volume should not exceed 1/10 of the total reaction volume.

2.5 Electrophoretic separation of nucleic acids

DNA was supplied with 1x DNA sample buffer and separated on a 1-2,5% agarose gel in 1xTAE-buffer. Ethidium bromide was incorporated in the agarose gel (1,5 µl of a stock solution in 150 ml gel solution) so that DNA bands were directly visible under UV-illumination. For separation of RNA, electrophoresis tanks and gel chambers were incubated with 3% H_2O_2 before use. RNA was diluted 1:1 with 2x RNA sample buffer containing ethidium bromide and denatured at 85°C for 5 to 10 min. After adding 6x RNA loading buffer, samples were separated on a formaldehyde-containing 1-1,5% agarose gel in 1x MOPS-buffer. For preparation of gels, agarose from Gibco-BRL (Invitrogen) was used.

2.6 Cloning

2.6.1 Plasmids

pCR 2.1-TOPO cloning vector	Invitrogen
pCR II-TOPO cloning vector	Invitrogen
pPCR-Script Cam SK(+) cloning vector	Stratagene
pBKSII-/Tst-1 (inserted POU-transcription factor Tst-1)	M. Wegner, ZMNH, Hamburg

2.6.2 Bacterial strains

TOP10 One Shot Chemically Competent E.coli cells	Invitrogen
Epicurian Coli XL10-Gold Kan ultracompetent cells	Stratagene

Both bacterial strains were used for amplification of recombinant DNA in form of plasmid-vectors. Selection occurred by addition of 100 µg/ml ampicillin or 10-20 µg/ml chloramphenicol, depending on the resistance gene in the particular plasmid. Broths were prepared as indicated in the list of frequently used buffers and solutions (section 2.15).

2.6.3 Insertion of DNA into plasmids

Linearised plasmid DNA was treated with shrimps alkaline phosphatase according to the manufacturer’s instructions in order to avoid re-ligation of vector ends in ligation assays. PCR cDNA fragments were added to linearised and dephosphorylated vector DNA in a 2:1 molar ratio and ligated over night with T4 DNA-ligase following the standard protocol described in (Sambrook and Russell, 2001), section 1.87. Alternatively, PCR fragments were ligated in 5-30 min. into pCR-TOPO cloning vectors with the TOPO TA cloning Kit according to the supplier's instructions.

2.6.4 Bacterial transformation

Competent bacterial cells were transformed chemically by heat shock (30 sec. at 42°C), grown for 1 hour in broth without antibiotic and then plated on agar plates containing 40µg/ml X-gal and the adequate antibiotic (20 µg/ml chloramphenicol and 100 µg/ml ampicilline, respectively). After incubation over night at 37°C, bacterial colonies could be selected by blue/white-screening. Insert-containing clones were white, whereas re-ligated vector clones without insert were blue.

2.6.5 Preparation of Plasmid DNA

Plasmid DNA, amplified in bacteria, was isolated and prepared with Miniprep- or Midiprep- Kits (Qiagen), corresponding to the expected quantities of plasmid DNA.

2.7 RNA Extraction and Purification

Total RNA was isolated from rat brain tissues (see also 2.14) using the RNeasy Mini Kit (Qiagen). Animal tissue was shock frozen in liquid nitrogen and disrupted and homogenized with a rotor-stator homogeniser in the presence of a denaturing guanidine isothiocyanate-containing buffer according to the manufacturer's instructions. Genomic DNA was digested with DNase I (Qiagen) as described in the RNeasy Mini Kit instructions. 10 mg of tissue yielded about 10-30 µg total RNA, depending on the developmental stage of the rats used. RNA quality was controlled on a denaturing agarose gel (see also 2.5); purity and concentration were determined by spectrophotometry at a wavelength of 260 nm.

2.8 Suppression Subtractive Hybridization (SSH)

The method of SSH, which enables researchers to compare two populations of mRNA and obtain clones of genes that are expressed in one population but not in the other, was originally described by (Diatchenko et al., 1996). In brief, mRNAs of both populations are converted into cDNA, hybridised with one another and the hybrid sequences removed. Consequently, the remaining unhybridised cDNAs represent genes that are expressed in one population but are absent from the other. In a following step, the differentially expressed sequences can be selectively amplified by PCR. The samples that were compared in the present analysis were derived from RNA from rat cerebellum at two developmental stages: embryonic day 18 (E18), corresponding to the time point of maximal axonal outgrowth of cerebellar Purkinje cells, and postnatal day 35 (P35), when axonal outgrowth of all cerebellar neurons is completed (approach “A”). A second approach (approach “B”) was similarly performed with RNA from rat entorhinal cortex at P0, when outgrowth of entorhinal fibers to the dentate gyrus of the hippocampus is maximal, and P10, when axonal outgrowth ceases. Complementary DNAs (cDNAs) were synthesized from 1 µg total RNA using the SMART PCR cDNA Synthesis Kit, and subtractive hybridisation was performed with the PCR-Select cDNA Subtraction Kit. cDNAs derived from the later stage of development, the reference cDNAs, were considered the “driver” pool, cDNAs derived from the earlier stage of development, which contained specific, differentially expressed transcripts, the “tester” pool.

cDNA-population of the subtraction approach	**Rat brain tissue**	**developmental age**	**characteristics**
“Tester” A	cerebellum	Embryonic day 18	Maximal outgrowth of Purkinje cell axons
“Driver” A	cerebellum	Postnatal day 35	Axonal outgrowth of all cerebellar neurons completed
“Tester” B	entorhinal cortex	Postnatal day 0	Maximal outgrowth of entorhinal perforant path fibers
“Driver” B	entorhinal cortex	Postnatal day 10	Entorhino-hippocampal pathway formation completed

Both cDNA samples were digested with the restriction endonuclease Rsa I to generate shorter, blunt-ended double-stranded cDNA fragments. To select for transcripts highly expressed in the “tester” population but strongly downregulated in the “driver” population, PCR adaptors were ligated to the “tester” pool, which then was hybridised with excess cDNAs from the “driver” pool. After hybridisation, suppression PCR with primers specific for the ligated “tester”-adaptors was applied for selective amplification of differentially expressed transcripts, present in the “tester” pool (cerebellum at E18 and entorhinal cortex at P0, respectively) but not present or expressed to a much lower level in the "driver" pool (cerebellum at P35 and entorhinal cortex at P10, respectively). Amplified cDNA sequences were then ligated into the pUC-derived TOPO TA-cloning vector pCR II-TOPO and transformed into E.coli bacteria. Subtracted clones were randomly picked, miniprepped and sequenced by capillary electrophoresis. Finally, all sequences were checked for homology to known genes in GenBank, EMBL, DDB and PDB databases using the following computer programs: BlastN and BlastP from the National Center for Biological Information, NCBI (www.ncbi.nlm.nih.gov/blast/), and NIX and PIX from the UK Human Genome Mapping Project Resource Centre (www.hgmp.mrc.ac.uk/Registered/Menu/). For sequences that gave significant similarities only to Expressed Sequence Tags (ESTs), overlapping EST-sequences were searched in the EST-divisions of GenBank, EMBL and DDB databases. Overlapping fragments were then compiled with the program Gene Runner (Version 3.00, Hastings Software, Inc.) and again used for homology searches to known genes.

2.9 In vitro transcription

Templates for in vitro transcription were generated either by PCR-amplification of DNA-sequences inserted into plasmids using T7- and sp6- promotor primers or by restriction digestion of plasmids containing the insert of interest. Single-stranded DIG-labelled cRNA probes were synthesized with T7- and sp6- RNA-polymerases, respectively, using a Digoxigenin RNA labelling mix (Roche) according to the manufacturer's protocol. DNA was digested with DNase I (Catalys) after completed riboprobe synthesis. After precipitation, DIG-labelled RNA probes were resuspended in ddH2O and RNase inhibitor was added (2-5 U/µl suspension volume). The concentration of synthesized riboprobe was estimated on a denaturing formaldehyde-containing agarose gel in comparison with defined quantities of a DIG-labelled control RNA (Roche).

2.10 Northern Blot analysis

5 µg of total RNA were size-fractionated on a 1,5% denaturing formaldehyde-agarose gel. After washing the gel 2 times in 20x SSC, a capillary blot was set up as previously described (Sambrook and Russell, 2001, section 7.35), and RNA was blotted onto a positively charged Nylon membrane (Roche). After UV-irradiation, the blot was incubated for 1h at 68°C in prehybridisation buffer. For hybridisation, 300 ng/ml riboprobe was added and the blot was incubated overnight at 68°C. The next day, the blot was washed twice in washing buffer 1 at room temperature for 15 min., followed by a washing step in the same buffer at 68°C for 30 min. Thereafter, the blot was washed twice in washing buffer 2 at 68°C for 30 min, blocked in 1% Blocking solution (Roche) for 45 min. at room temperature and incubated 1h with anti-DIG-alkaline phosphatase-conjugated Fab

fragments (Roche, 1:10000) at room temperature. Prior to detection, the blot was washed 3 times with washing buffer 3. Chemiluminescent detection was performed with CSPD-substrate for alkaline phosphatase (Roche) according to the manufacturer’s instructions and light emission was recorded on an X-ray film. To re-hybridise blots with another riboprobe, chemiluminescence was removed with water and blots were stripped twice in 0,2% SDS just before boiling for 10 min. After washing in washing buffer 4 for 10 min., blots were prehybridised and hybridised as before. Any changes in the performance are specified in the appropriate text part in chapter 3.

2.11 In situ-Hybridisation

Embryonic and neonatal Sprague-Dawley rats were decapitated, their brains removed, rapidly frozen in tissue freezing medium and kept at –80°C until use. Starting at 5 weeks of age, Sprague-Dawley rats were anaesthetized with 0,1 ml/100 g body weight vetanarcol before decapitation. In situ hybridisation was performed as described previously by Schaeren-Wiemers and Gerfin-Moser with slight modifications: 20 µm-frozen sections were cut on a cryostat, mounted on superfrost plus slides and dried on a hot plate at 45 °C for 1h (Schaeren-Wiemers and Gerfin-Moser, 1993). Sections were then incubated in fixative for 10 min., washed 3 times in PBS and afterwards treated with acetylation solution for 10 min. Sections were then again washed 3 times in PBS. Prior to hybridisation, sections were preincubated for 1h at room temperature in prehybridisation buffer. Hybridisation was carried out overnight in prehybridisation buffer with 500 ng/ml of either antisense or sense digoxigenin-labelled riboprobe at 72°C in probe-clip press-seal incubation chambers (Sigma). Tissue sections were subsequently incubated in 0,2x SSC at 68°C for 1h and rinsed with 0,2x SSC at room temperature thereafter. Following a short wash in Maleic acid buffer, sections were blocked in 1% blocking solution (Roche) for 1h at room temperature, shortly rinsed in maleic acid buffer and incubated with anti-DIG-alkaline phosphatase-conjugated Fab fragments (Roche, 1:5000) for 1h at room temperature. After a short incubation in DIG-detection buffer, colour detection of transcripts was carried out upside down in DIG-colour reaction solution. After stopping the enzymatic reaction in TE-buffer, sections were cover slipped with Kaiser’s Glycerine gelatine. Cresylviolet staining was performed after fixation and PBS washes, sections were cover slipped with Eukitt (O.Kindler GmbH, Freiburg, Germany). Stained brain sections were digitally photographed with a Spot camera (Diagnostic Instruments) mounted on an Olympus AX70 microscope. Any changes in the performance are specified in the appropriate text part in chapter 3.

2.12 Equipment

Cryostat (CM1900)	Leica
ABI PRISM 310 genetic analyser (capillary sequencer)	Applied Biosystems
Balances	Ohaus and Mettler-Toledo
UV-spectrophotometer	Applied Biosystems
Electrophoresis tanks and power supplies	Owl
Power supplies	Apelex, BioRad
Centrifuges (Biofuge stratos, Megafuge 1.0, Biofuge pico)	Heraeus
Bacterial Incubator	Heidolph
Hybridisation oven	Heraeus
Thermomixer	Eppendorf
Gel Documentation System	Intas
Microscope (AX70)	Olympus
Camera	Diagnostic Instruments Inc.
Rocking platfom	Heidolph
Ultra-Turrax T50	IKA
Magnetic Stirrer	IKA
PCR-Mastercycler gradient	Eppendorf
GeneAmp PCR system 2400	Applied Biosystems

2.13 Consumables

2.13.1 Chemicals

Unless otherwise annotated in the text, chemicals were obtained from Merck, Fluka, Axon Lab, Sigma, AppliChem and Roth.

2.13.2 Enzymes

Restriction endonucleases	Roche
Shrimps alkaline phosphatase	Roche
T4 DNA-ligase	Roche
RNase-inhibitor	Invitrogen

T7-RNA-polymerase	Roche
Sp6-RNA-polymerase	Roche
PowerScript Reverse Transcriptase	Clontech
Expand Reverse Transcriptase	Roche
PWO DNA-polymerase	Roche
Taq DNA-polymerase	Roche
Advantage 2 Polymerase Mix	Clontech
DNase I, RNase-free	Catalys / Promega and Qiagen

2.13.3 Materials

Nylon membranes, positively charged	Roche
X-ray films	Roche
Hybridisation Bags	Roche
Super Frost Slides	Menzel-Gläser
Cover Slips	Menzel-Gläser
Jung Tissue Freezing Medium	Leica
Kaiser's Glycerine Gelatine	Merck

2.13.4 Kits

ABI PRISM BigDye Terminators Cycle Sequencing Ready Reaction Kit	Applied Biosystems
Qiaprep Spin Mini- and Midiprep Kits	Quiagen
Qiaquick PCR Purification Kit	Qiagen
Qiaquick Gel Extraction Kit	Qiagen
RNeasy Mini Kit	Qiagen
PCR-Select cDNA Subtraction Kit	Clontech
SMART PCR cDNA Synthesis Kit	Clontech
DIG Luminescent Detection Kit	Roche
TOPO TA Cloning Kit	Invitrogen
PCR-Script Cam Cloning Kit	Stratagene

2.13.5 Oligonucleotide primers [for sequencing or amplification of DNA-fragments by Polymerase chain reaction (PCR)]

All primers were designed using the computer program Gene Runner, Version 3.0 (Hastings Software Inc.) and purchased from MWG-Biotech. Degenerate primers specific for different classes of transcription factors were designed based on the conserved regions in the DNA-binding domain of transcription factor families. They are specified in detail in the results-section 3.1.1.

Other oligonucleotide primers (nucleotide sequence is shown in 5'→3' direction):

T7-backward primer: TAATACGACTCACTATAGGG
sp6-forward primer: TATTTAGGTGACACTATAG
sp6-forward primer/TOPO: TATTTAGGTGACACTATAGTCGGATCCACTAGTAACG
rat-CRHSP-forward: GCCATGTCATCTGAACCTC
rat-CRHSP-backward: TCCTTAGGAGCTGATGACG
anchor-primer 3'-RACE: GACCACGCGTATCGATGTCGAC
race-zinc1A: GGGCTACACTCTGAACTCGAATCT
race-zinc1B (nested): TCCAGGTCCATTTGCGAGTCCAC
T7-zfp-mouse-forward:
AAATTTAATACGACTCACTATAGGGAGGACGCTGTACCAAGATG
sp6-zfp-mouse-backward:
ATTTTAATTTAGGTGACACTATAGAACTTGGTGAGGATCCGAGCTGG
rat-zfp-1/backward: ACGGGTTCCCCAGTGCTTTTAC
mouse-zfp-forward: CGAGGGAGAGAGATTCAGGGATTAAC
mouse-zfp-backward1: GCCCTCCCTCCTGAGTCTAACACTTG
mouse-zfp-backward2: TCGGTAACGGCTTCTCTG

2.14 Animals

Sprague Dawley rats, strain OFA	Charles River Laboratories Inc.

2.15 Frequently used buffers and solutions

Solutions used for work with RNA were prepared in ddH_2O, treated with DEPC (0,1%) and autoclaved or, alternatively, were directly prepared in DEPC-treated and autoclaved ddH_2O.

1x TAE (Tris acetate-EDTA)	40 mM Tris-Acetate 1 mM EDTA

TE-buffer	10 mM Tris-HCl, pH 8.0 5 mM EDTA
1x PBS, pH 7.4	140 mM NaCl 2,7 mM KCl 10 mM Na2HPO4 1,8 mM KH2PO4
6x DNA sample buffer	0,25% (w/v) Bromophenolblue 0,25% (w/v) Xylene Cyanol 30% glycerol
2x RNA sample buffer	250 µl deionised formamide 100 µl 37% formaldeyde 100 µl 5x MOPS 1 µl Ethidium bromide (10 mg/ml)
6x RNA loading buffer	0,5% SDS 25% glycerol 25 mM EDTA 250 µg/ml bromophenolblue solution
RNA agarose gel (1,5%, 150 ml)	2,25g Agarose 25 ml 5x MOPS 100 ml ddH$_2$O boil in a microwave, then cool to 60°C add 25,5 ml 37% formaldehyde
5x MOPS	0,2 M MOPS 50 mM Sodium-Acetate, pH 7.0 10 mM EDTA adjust to pH 7.0 with NaOH store light protected at 4°C
Maleic acid Buffer	0,1 M Maleic Acid, pH 7.5 0,15 M NaCl
20x SSC	175,3 g NaCl 88,2 g Na-Citrate (trisodium-citrate dihydrate) dissolve in 800 ml H$_2$O adjust pH to 7.0 with NaOH fill up to 1l

Fixative	4% Paraformaldehyde in 1x PBS add few drops of 1N NaOH heat to 65°C for dissolving filter
Acetylation solution	0,1 M triethanolamine add 0,25% acetic anhydride just before use
Prehybridisation buffer (in situ-Hybridis.)	50% formamide 5x SSC 5x Denhard's Solution (Sigma) 100 µg/ml sheared herring sperm (Roche) 250 µg/ml baker's yeast tRNA (Sigma)
Prehybridisation buffer (Northern Blot)	50% formamide 5x SSC 2% blocking solution (Roche) 0,02% SDS 0,1% N-Lauroylsarcosine
DIG-Detection buffer	100mM Tris/HCl, pH 9.5 100 mM NaCl
DIG-Colour reaction solution	10 ml DIG-Detection buffer 45µl NBT (75 mg/ml in 70% DMF) 35 µl BCIP (50 mg/ml in 100% DMF) 100-µl levamisol (24 mg/ml Detection buffer)
Washing buffer 1	2x SSC 0,1% SDS 0,5% Blocking Reagent
Washing buffer 2	0,1% SSC 0,1% SDS
Washing buffer 3	0,1M Maleic Acid, pH 7.5 0,15M NaCl 0,3% Tween 20
Washing buffer 4 (after stripping)	0,1 M Maleic Acid, pH 7.5 0,15 M NaCl 3% Tween 20
LB-broth	1% Tryptone 0,5% Yeast Extract

	1% NaCl adjust pH to 7.0 with NaOH and autoclave add antibiotic after cooling to 55°C (100 µg/ml Ampicillin or 10-20 µg/ml Chloramphenicol)
LB-agar	1% Tryptone 0,5% Yeast Extract 1% NaCl adjust pH to 7.0 with NaOH 1,5% Bacto-Agar autoclave, add antibiotic after cooling to 55°C
SOC-broth (for bacterial transformation)	2% Tryptone 0,5% Yeast Extract 10 mM NaCl adjust pH to 7.5, autoclave and add: 2,5 mM KCl 10 mM MgCl2 10 mM MgSO4 20 mM sterile glucose

3 Results

3.1 Identification of transcription factors in developing rat cerebellum and entorhinal cortex

The present approach aimed at the identification of transcription factors in rat cerebellum and entorhinal cortex, respectively, at the developmental age when extensive axonal outgrowth occurs in these brain regions. It was based on the method of “degenerate PCR” that employs degenerate primers for PCR amplification, which are specific for a conserved DNA region common to members of a certain class of molecules.

3.1.1 Classes of transcription factors and generation of degenerate primers

Transcription factors are proteins that specifically bind to DNA and initiate transcription of certain genes. In principle, they consist of two different domains, a DNA-binding domain and a transactivation domain. The latter interacts with other proteins of the transcription complex and thereby assembles them at the promotor site of a certain gene to start its transcription. Both domains are separated by a variable spacer region. Some transcription factors additionally contain a dimerization domain, which is necessary for the formation of transcription factor homo- or heterodimers. Depending on the structure and sequence of the DNA-binding domain, several classes of transcription factors can be distinguished. Within one class, the DNA-binding domains are highly conserved on the amino acid level. (*fig. 3-1*). In order to isolate transcription factors by PCR amplification, degenerate primers were generated on the basis of conserved DNA-binding domains of respective transcription factor classes (*fig. 3-1*). cDNA from either cerebellum at embryonic day 18 (CE-E18) or entorhinal cortex from neonatal rats (EC-P0) was taken as template for PCR amplification with degenerate primers. cDNA-synthesis was carried out by reverse transcription from 1 µg total RNA extracted from CE-E18 and EC-P0, respectively.

POU:	L E Q F A K Q F K Q/H	
degen. primer:	CTG GAR CAR TTY GCY AAR CAR TTC AAG CA	
	V W F C N R R Q K	
degen. primer:	GTN TGG TKT TGT AAC AGR AGR CAA AAA	
HOX:	E L E K E F/L	K I W F Q N R R
degen. primer:	GAR CTN GAR AAR GAR TT	AAR ATH TGG TTY CAR AAY CGN CG
Zinc finger:	C X X C G K	H T G E R/K P Y
degen. primer:	TGY CCN GAR TGY GGN AA	CAY ACD GGH GAR CGY CCN TAY
bHlH:	R E R X R	Y I D/E A/V L
degen. primer:	MGR GAR CGS MRS MG	TAY ATC GAS GYY CT
ETS:	W Q F F/L F/L D/R	N/K M N Y D/E K
degen. primer:	TGG CAR TTY YTN YTN SA	AAN ATG AAY TAY SAN AAG
Forkhead:	Y I A L I T/A M A I	
degen. primer:	TAY ATH GCN CTN ATY RCN ATG GCN AT	
	N S I R H N/S L S	
degen. primer:	AAY AGY ATH CGN CAY ARY CTN TC	

fig. 3-1: **Conserved domains of selected transcription factor classes and primer design for PCR-amplification of these regions with degenerate primers.**
In bold on the left side are given the different transcription factor classes. Amino acids of conserved DNA-binding domains are highlighted in red behind each transcription factor class. „N“ stands for any amino acid. The consensus amino acid sequence was taken as a basis to generate degenerate primers in order to amplify the class-specific domains by PCR. Primer sequences are shown as nucleotide triplets in 5’→3’ direction under the corresponding amino acid domain. Degenerate forward primers are underlined by a continuous line, degenerate backward primers by a dotted line. The following abbreviations were used for the selection of multiple nucleotides: R=A/G; Y=C/T; S=G/C; W=A/T; M=A/C; K=G/T; B=C/G/T; D=A/G/T; H=A/C/T; V=A/C/G; N=A/C/G/T.

3.1.2 PCR-amplification of the conserved POU-domain

The POU family is a group of related transcription factors with a particular type of bipartite DNA-binding domain, containing the POU-specific domain and the POU-homeodomain (*fig. 3-2*). In order to amplify the DNA-binding domain by PCR from different transcription factors of the POU family, a forward primer was generated from the POU-specific domain and a backward primer from the POU-homeodomain.

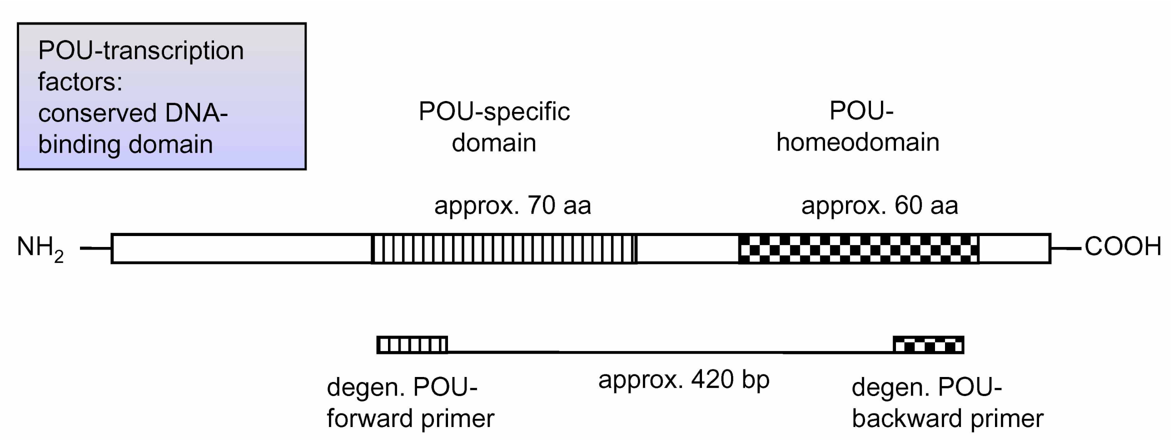

fig. 3-2: **schematic drawing of the conserved DNA-binding domain in POU-class transcription factors.**
The bar represents the amino acids of any given POU-transcription factor. The POU DNA-binding domain comprises a POU-specific domain (highlighted by stripes) and a POU-homeodomain (highlighted by little squares), which is located further to the c-terminus. For PCR-amplification of POU-transcription factors, a degenerate forward primer was chosen from the POU-specific domain and a degenerate backward primer from the POU-homeodomain. As both domains as well as the spacer region in between are fairly constant in length, the amplified PCR product is predicted to consist of approx. 420 bp.

All PCR amplifications using degenerate POU-primers were performed as follows:

initial denaturation step:	94°C – 2 min.	
denaturation:	94°C – 30 sec.	33x
primer-annealing:	45°C – 45 sec.	
elongation:	72°C – 1min.	
final elongation step:	72°C – 7 min.	

To confirm the specific amplification of the POU-domain with the degenerate POU-primers, a plasmid, in which the POU domain gene Tst-1 was inserted (pBKSII-/Tst-1), was used as a control template in the PCR approach. A distinct DNA-fragment of approx. 420 bp was amplified in the control (*fig. 3-3*, *lane 1*), indicating that the PCR conditions were set up correctly. Likewise, a distinct PCR product of 420 bp was detectable in the reaction using cDNA from embryonic cerebellum as template (*fig. 3-3*, *lane 2*). In a subsequent PCR, this product was specifically re-amplified using an aliquot of the first reaction as template and the same primers for amplification (*fig. 3-3, lane 3*). This confirmed the specificity of the POU-fragment amplified in the first experimental reaction. The size of the PCR-product (420 bp) corresponded with the predicted size of the DNA-fragment. A certain variability in size occurs due to the variable linker region between the POU DNA-binding domain and homeodomain.

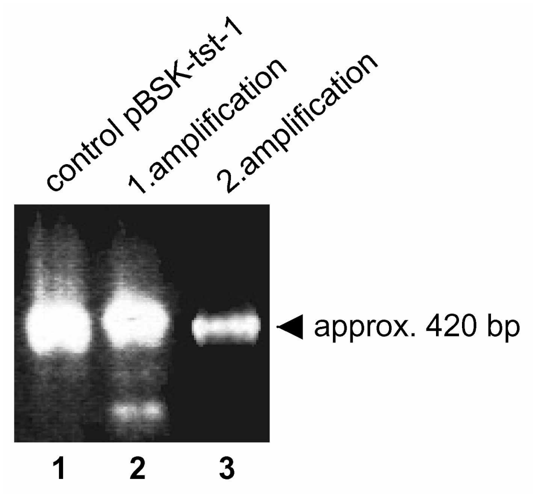

fig. 3-3: **PCR amplification of the conserved POU DNA-binding domain in rat embryonic cerebellum using degenerate POU-primers.**
To amplify a 420 bp fragment of the conserved POU DNA-binding domain, the following templates were employed: *lane 1:* 200 ng control plasmid pBKSII-/Tst-1, in which the POU-transcription factor Tst-1 is inserted, *lane 2:* 5 µl cDNA, which was reverse transcribed from 1 µg total RNA from rat E18-cerebellum (first amplification), *lane 3:* 0,5 µl of the first amplification product shown in lane 2 (re-amplification).

In the entorhinal cortex of newborn rats, a 420 bp POU-fragment was similarly amplified by PCR with degenerative primers (data not shown).

The 420 bp band was extracted from the agarose gel, purified and cloned into the pCR-Script CAM cloning vector (Stratagene). This plasmid carries a chloramphenicol resistance gene, which allows transformed bacteria to grow in the presence of the antibiotic choramphenicol. XL1-Blue bacteria were transformed with this construct, selected for chloramphenicol resistance and the amplified plasmid DNA of growing colonies was subsequently isolated by mini-prep (Qiagen) and sequenced. Sequence analysis of 35 inserted cDNA fragments was performed with the computer programs *Chromas* and *BlastN* by the blastn and tblastn sequence alignment algorithms (Altschul et al., 1990). In 26 cases a homology to two known rat POU class III transcription factors, brain-2 and brain-4, was observed. The residual 9 clones did not contain any insert or produced defective sequence results. Whereas the brain-4 cDNA fragment was amplified by degenerate POU primers in cerebellum as well as in entorhinal cortex, the brain-2 cDNA fragment was isolated only from entorhinal cortex. As function and expression patterns of both proteins are well examined (He et al., 1989; Le Moine and Young, 1992; Mathis et al., 1992; reviewed by Rosenfeld, 1991) and no further cDNA-fragments from different POU- transcription factors were amplified, the following PCR approaches with degenerate primers concentrated on other transcription factor classes.

3.1.3 PCR-amplification of the conserved C_2H_2-Zinc finger domain

In most zinc finger transcription factors, the conserved zinc finger domain (*fig. 3-4*) does not exist in a single form but is arranged in repeats with short spacer regions in between. As a consequence, degenerate zinc finger primers can bind to more than only one site in the conserved region. This results in amplification of multiple PCR products as illustrated in *fig. 3-4*.

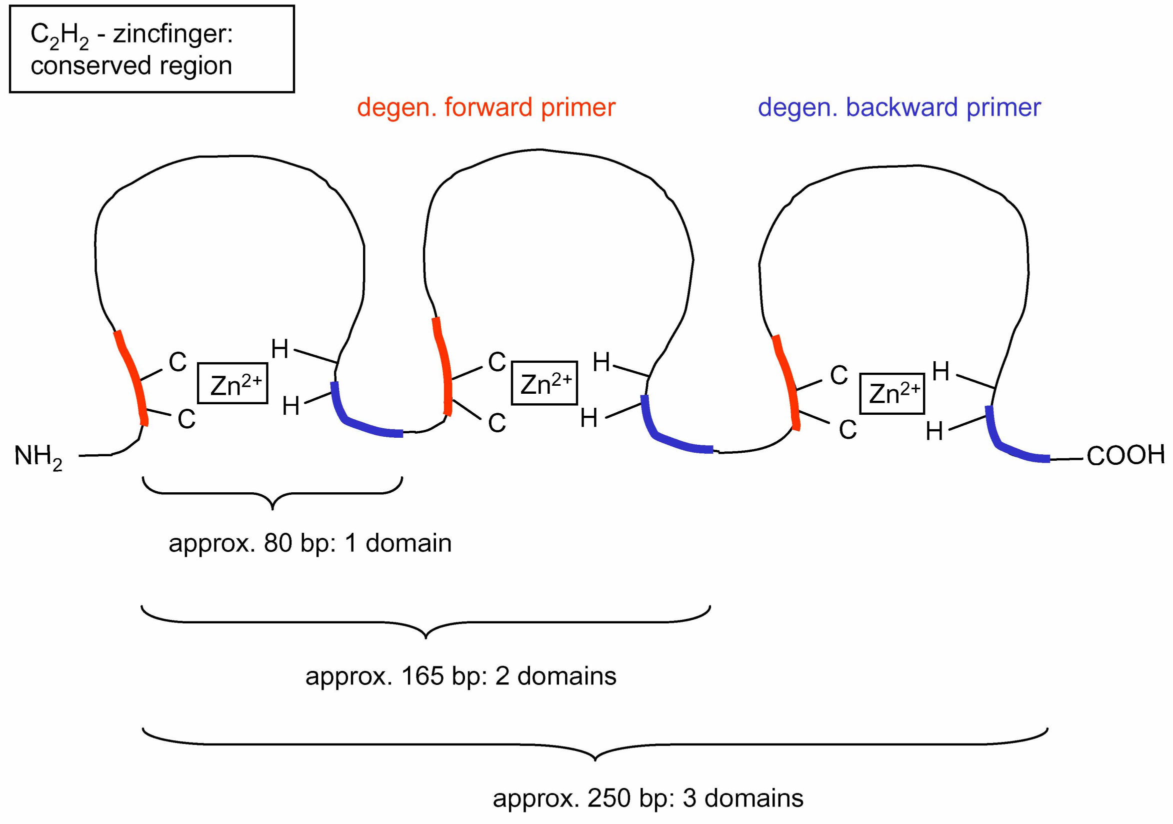

fig. 3-4: Schematic illustration of the conserved C_2H_2-zinc finger domain.
The line represents the amino acid sequence of any given C_2H_2-zinc finger transcription factor. Bulges indicate the tertiary structure of the protein, which occurs by complexing a zinc ion by two conserved cysteine (C) and two histidine (H) residues. As this domain is often arranged in a repeated pattern degenerate zinc finger primers are able to bind the DNA of such a protein at multiple sites. They therefore can generate several amplification products, a ladder of bands, depending on the number of domains present in the zinc finger protein. The smallest amplified unit corresponds to a single domain, which is approx. 80 bp in length. Each additional domain will result in the amplification of a PCR product, which is approx. 85 bp longer.

Cycling parameters for PCR-amplification of C_2H_2 –zinc finger domain transcription factors with degenerate zinc finger primers were as follows:

initial denaturation step:	94°C – 2 min.	
denaturation:	94°C – 30 sec.	30x
primer-annealing:	42°C – 45 sec.	
elongation:	72°C – 1 min.	
final elongation step:	72°C – 7 min.	

Fig. 3-5 shows the amplification of zinc finger regions in rat cerebellum (CE-E18, *lane 1*) and entorhinal cortex (EC-P10, *lane 2*). The degenerate zinc finger-PCR resulted in multiple amplification products of one or more fingers in form of a ladder. In cerebellum and entorhinal cortex, the largest amplified fragment was 510 bp in length, correlating to six zinc finger domains.

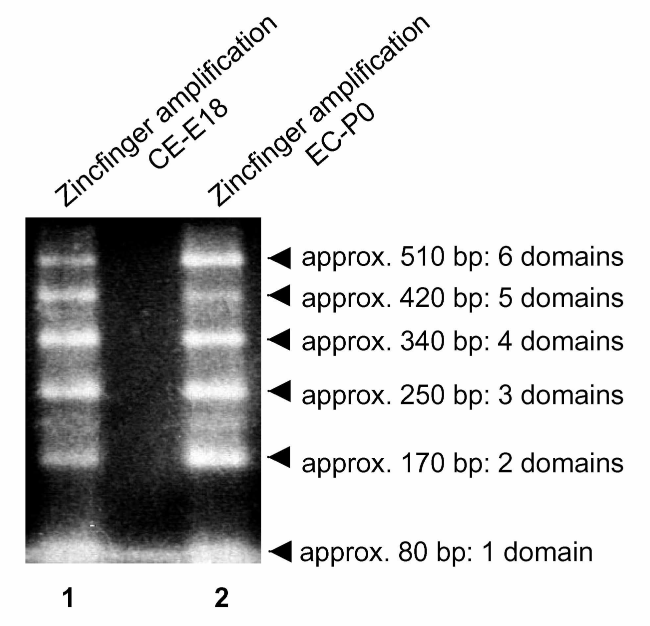

Fig. 3-5: PCR amplification of C_2H_2-zinc finger domains in rat cerebellum at E18 and in entorhinal cortex of neonatal rats.

1 µg of total RNA from either E18-cerebellum or P0-entorhinal cortex were reverse transcribed and used in a PCR with degenerate zinc finger primers. Multiple PCR products were amplified, corresponding to the number of zinc finger domains present in a single zinc finger protein. The PCR fragments correspond to the calculated sizes of consecutively positioned zinc finger domains (see fig.3-4).

PCR-amplified zinc finger cDNAs that contained at least 3 conserved zinc finger domains were excised and eluted from the agarose gel and cloned into the pCR-Script CAM Cloning Vector from Stratagene. Constructs were transformed into XL1-Blue bacteria, which were then selected for choramphenicol resistance. 24 bacterial clones were selected for amplification and plasmid preparation. The cloned PCR-fragments were sequenced and analysed with the computer programs *Chromas* and *BlastN*. 19 of all sequenced cDNAs showed homologies to zinc finger proteins from other species. Remaining clones contained unknown cDNAs. However, as the amplified PCR products consisted almost exclusively of conserved zinc finger domains, they could not be used as tools for the further characterization of these genes. In particular, a certain amount of gene-specific sequence information outside the conserved region was necessary for a good comparison of the cloned gene fragments with zinc finger proteins from other species and to generate gene-specific probes for subsequent expression analyses. Therefore, the RACE-method was applied which allows a selective elongation of either the 3'-or the 5'-end of any given sequence of an unknown gene. A cDNA sequence, which contained 3 zinc finger domains and showed homology to a mouse zinc finger protein was chosen for RACE-elongation. In the following, the gene and protein for this cDNA were named zfp-1. Two forward-primers were generated for this clone, both binding specifically between two conserved zinc finger domains ('primer 1' and a further 3'-binding primer 'nested primer 2'). An approx. 330 bp fragment was amplified in a first 3'-RACE-PCR using 'primer 1' and was re-amplified in a second RACE-reaction with 'nested primer 2' (*Fig. 3-6*). After elution from the agarose gel, the fragment of interest was cloned and the DNA sequenced. Sequence analysis resulted in the identification of the 3'-cds of zfp-1.

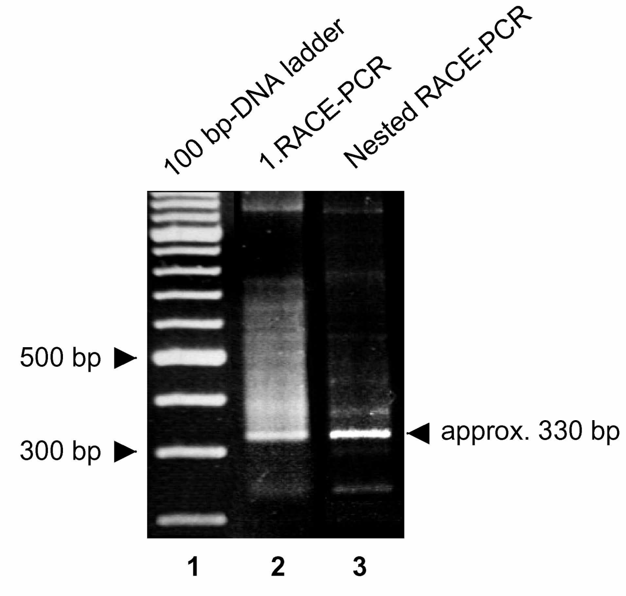

Fig.3-6: **3'-RACE (Rapid Amplification of cDNA ends) with the 3 zinc finger-domain containing cDNA zfp-1.**

A PCR-amplified cDNA from CE-E18, containing 3 zinc finger domains and named zfp-1, was elongated at its 3'-end by 3'-RACE-PCR. A first RACE-PCR resulted in an approx. 330 bp cDNA fragment (*lane 2*), which was clearly re-amplified in a nested RACE-PCR (*lane 3*). A DNA size marker was loaded on *lane 1*.

Both sequences for zfp-1, the first obtained by degenerate zinc finger-PCR and the second obtained by 3'-RACE, were assembled at their overlapping regions with the help of the computer program *Gene Runner*. This resulted in a total sequence length of 527 bp of the identified rat zfp-1 clone, containing 5 conserved zinc finger regions and the 3'-end of the coding region. Using this sequence, a new search in the GenBank database was accomplished in order to find homologous sequences from other species and overlapping rat EST-clones. A zinc finger protein from the mouse, Zfp61 (accession numbers: L28167 and NM_009561) displayed strong homology to the identified zinc finger protein zfp-1 in the rat (88% identity of nucleotides). The next step aimed at confirming the homology between rat zfp-1 and the mouse zinc finger protein outside the conserved zinc finger region. A forward and backward primer were therefore generated from the non-conserved 5'-cds of the mouse zinc finger protein Zfp61. Based on the strong homology found in the conserved 3'-cds between mouse and rat, these primers were used in a PCR with rat brain tissues from different developmental stages (*Fig. 3-7*). PCR yielded an approx. 600 bp-product - corresponding to the calculated size from the mouse sequence - which delivered new sequence information about the 5'-cds of rat zfp-1. Comparison of the 600 bp-sequence with the homologous sequence span in mouse resulted in 80% identity on the nucleic acid level. Conservation of the nucleic acid sequence was thus similar in the conserved and in the non-conserved region between both species.

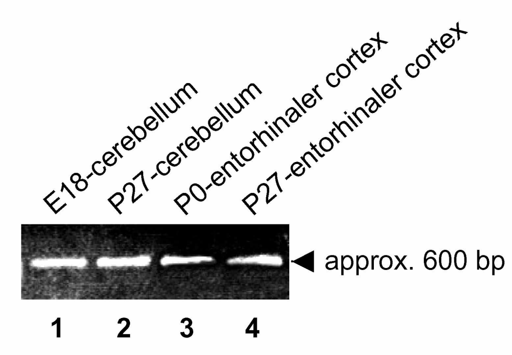

Fig. 3-7: **PCR-amplification of the 5'-region of the coding sequence (cds) of rat zfp-1.**
The forward primer was generated from the 5'-region of the mouse homologue of rat zfp-1. It was used in a PCR reaction together with a backward primer, generated from the RACE-elongated 3'-end of rat zfp-1. cDNA from rat cerebellum or entorhinal cortex at different developmental stages was taken as template for PCR. The PCR product, which was approx. 600 bp in size, was amplified in cerebellum and entorhinal cortex at different developmental stages.

In order to generate DIG-labelled probes for expression studies by non-radioactive in situ-hybridisation, two primers were designed which were specific to the non-conserved region of rat zfp-1 and to which promotor sites for T7- and sp6- RNA-polymerases were attached. This allowed synthesizing DIG-labelled riboprobes directly from the PCR product. The riboprobes were used for analysis by in situ-hybridisation on rat sagittal brain sections. Several different concentrations of antisense and sense riboprobe were used. Although many parameters of the protocol for in situ-hybridisation were varied, the sense probe always yielded in hybridisation patterns similar to the antisense probe. It therefore was impossible to analyse the expression pattern of zfp-1 in rat brain during early postnatal development. Because of the extreme time-consuming approaches required for generation of zinc finger gene-specific sequences outside the conserved domain it was decided to not further study up the identified zinc finger containing cDNAs.

3.1.4 PCR-amplification of the conserved HOX-, ETS-, Forkhead- and bHLH-domain

Using degenerate primers for the HOX-, ETS-, Forkhead- and bHLH- transcription factor classes in PCR approaches, no specific PCR products were amplified. Plasmids that contained the cDNA for a specific transcription factor of each class were used as control templates in the PCR. With none of these it was possible to amplify the conserved domain using the adequate degenerate primers. PCR always produced multiple bands, none of which corresponded to the size of the conserved transcription factor domain. Most probably, the degeneration of the primers was too high resulting in unspecific primer binding and amplification of unspecific cDNA fragments.

3.1.5 Short Résumé

The original project of identifying transcription factors associated with axonal growth based on PCR amplification with degenerate primers resulted in the cloning of several cDNAs representing transcription factors from the POU and zinc-finger families. For the other transcription factor families it was not possible to design primers with a sufficient degree of degeneration to allow the identification of novel family members because the degenerate primers did not result in the amplification of any PCR products. With the POU-specific primers only two POU genes were identified which were already extensively studied and were unlikely to play a major role for axonal outgrowth. The most promising results were obtained with the zinc finger specific primers, which yielded a number of cDNAs coding for zinc finger domains, not represented in the database. Due to the structure of the zinc finger proteins and the location of the primers it was not possible to amplify cDNAs outside the zinc finger domains. Due to the high conservation of these domains it was not possible to use these probes for further analysis of the genes, e.g. by Northern blotting or in situ-hybridisation. For the generation of a meaningful cDNA probe it was necessary to extend the cDNA by the RACE procedure. Unfortunately, it was very time-consuming to generate cDNAs by 3’-RACE specific for originally amplified zinc finger cDNAs. In the one example where the procedure worked successfully the generated probe still was not appropriate for in situ hybridisation experiments. For these reasons it did not appear to be promising to further pursue this approach. Another strategy was therefore started which was methodically different but followed a similar goal. In order to identify genes that are highly expressed in late embryonic or neonatal brain in regions where massive fiber outgrowth occurs at that time but which are downregulated after cessation of axonal outgrowth, a subtractive hybridisation approach was performed with two rat brain tissues, embryonic cerebellum and neonatal entorhinal cortex. Compared to the first approach, the aim of the new strategy was to increase the number of molecules to be further analysed and to reduce the number of molecules that are equally expressed during development of the CNS and in the adult brain. Thus, the selectivity for transcription factors was given up, and the scope was widened to all classes of molecules that are developmentally regulated during the postnatal decrease of axonal growth competence.

3.2 Identification of genes developmentally regulated in postnatal rat brain during neuronal differentiation

3.2.1 Suppression Subtractive Hybridisation with rat cerebellum at two developmental stages

The age-dependent decrease in axon growth potential is well studied in the rat cerebellum *in vitro* and occurs in the early postnatal period around postnatal day 7 (P7) in cerebellar Purkinje cells (Dusart et al., 1997). This study served as a basis to determine the time points for the cerebellar subtraction approach: embryonic day 18 (E18), corresponding to the time point of beginning differentiation of cerebellar Purkinje cells, and postnatal day 35 (P35), when axonal projections of all cerebellar neurons are established. By subtraction of CE-P35 from CE-E18 transcripts, genes that are stronger expressed at E18 and which are downregulated at P35 were enriched. *Fig. 3-8* demonstrates the differential PCR amplification patterns of transcripts from rat cerebellum at E18 before and after subtraction. As expected, clear differences in the banding patterns were visible: some amplification products that were present before subtraction had disappeared or were more faint after subtraction, others were distinct after subtraction but could not be discerned beforehand.

The PCR amplified cDNAs were cloned and the yield of the generated subtractive library was roughly estimated by evaluating the total number of white bacterial colonies grown under antibiotic selection and considering the number of clones containing defective inserts or being present in multiple copies. About 800 independent clones were calculated for the cerebellar subtraction approach. Of those, 65 clones were randomly chosen for sequence analysis. By comparison with the databases, 30 clones were found to be identical to known rat genes or highly similar to homologues in other species. 7 sequenced cDNA fragments were completely unknown and 4 clones corresponded to rat EST sequences. Six sequences were found twice. Remaining clones (18) did not contain a specific insert or produced defective sequence results.

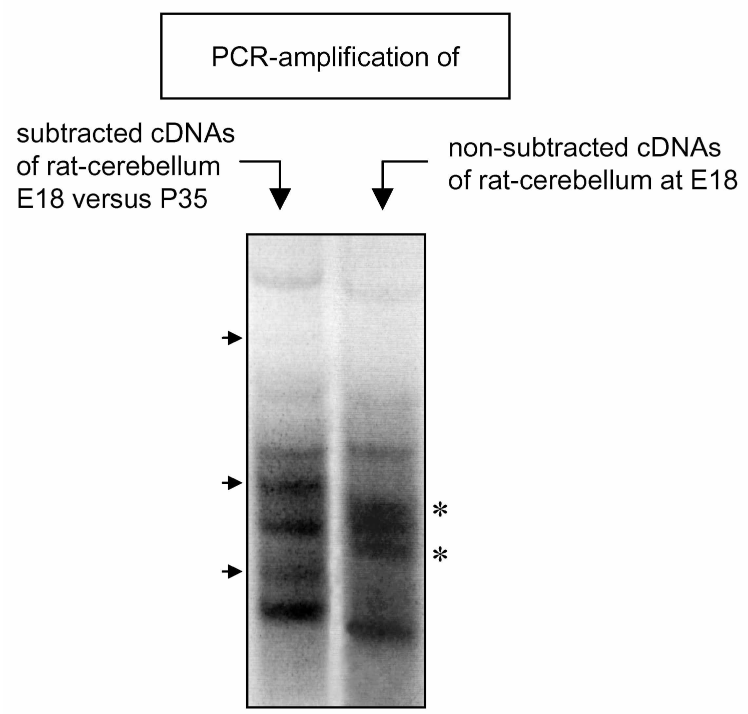

Fig. 3-8: Enrichment of transcripts present in rat cerebellum at E18 by suppression subtractive hybridisation

Comparison of gene expression profiles from rat cerebellum at E18 and P35. Transcripts more abundant at E18 than at P35 were enriched during the subtraction approach, whereas transcripts equally present in both populations were suppressed. In a final PCR step, enriched transcripts were amplified (left lane). As a control, PCR-amplification of all transcripts from E18-cerebellum before subtraction is shown (right lane). Pronounced differences between both lanes are visible: as an example, some enriched cDNAs (distinct after subtraction in the left lane) are marked by an arrow, suppressed cDNAs (more abundant before subtraction in the right lane) are marked by an asterisk.

Proteins encoded by known genes were classified into 6 groups: (1) transcription factors and other nuclear proteins, (2) cytoskeletal proteins, (3) signalling molecules, (4) enzymes, metabolic proteins and transporters, (5) cell surface molecules and (6) growth associated proteins. This classification is shown in *table 3-1*. For some of the subtracted genes data are present in the literature showing that they are downregulated in particular brain regions during development on either transcript or protein level. These genes are indicated by an asterisk (*) in *table 3-1*. This confirms that the used subtraction method indeed enriched for differentially regulated genes that are expressed more abundantly in developing than in mature CNS. The generated subtractive cerebellar cDNA library thus provides a reliable tool to search for possible candidate molecules that might play a role in neuronal differentiation.

Classification groups	*Protein products of identified genes*
transcription factors and other nuclear proteins	human TAR-DNA-binding protein TDP-43
	human zinc finger protein BCL11A
	rat cyclin D2
	human / mouse DYRK-kinase 2
	rat U5-snRNP
	mammalian basal Histone protein H2A.Z
	hnRNP R
	rat helix destabilizing protein hnRNP A1
	human nuclear import receptor snurportin-1
cytoskeletal proteins	rat nestin *
	rat class I β-tubulin *
	rat cytoplasmic form of χ-actin
	rat α-tubulin
signalling molecules	rat ras-GTPase activating protein ras-GAP
	mouse quaking gene product QKI-7
	rat calcium-regulated heat stable protein CRHSP-24
enzymes, metabolic proteins and transporters	human GPI transamidase
	rat ubiquitin carboxyl-terminal hydrolase PGP 9.5
	rat heme-oxygenase 2
	mouse WRS tryptophan tRNA-ligase
	human peroxisomal integral membrane protein PMP34
	rat differentiation-assoc. phosphate cotransporter DNPI
	rat cytochrome c
cell surface molecules	rat neuronal surface glycoprotein MRC OX-2 *
	rat neurotrimin *
	rat CD24 *
	rat HB-GAM / pleiotrophin *
growth associated proteins	rat thymosin-β4 *
	rat SCG-10 *
	rat cytosolic phosphoprotein p19 / stathmin *

Table 3-1: Classification of protein products of genes enriched in a subtractive library of rat cerebellum at E18.

Specified in this list are those clones identified in the subtraction which are identical to known rat genes or which displayed a strong similarity to homologous genes in mouse or human (30 of 65 sequenced clones). Proteins for which data in the literature show that they are downregulated during brain development either on transcript or protein level are indicated by an asterisk (*).

3.2.1.1 Identification of downregulated CRHSP-24 gene expression during brain development

One of the clones (clone 11) identified in the cerebellar subtraction approach was about 560 bp long and showed striking homology to the 3´-untranslated region (UTR) of mouse calcium-regulated heat stable protein CRHSP-24 gene (accession number: AK004711). In the database, the corresponding homologue in rat was found. This sequence (accession number AF115346) consists of the complete coding sequence (cds) for rat CRHSP-24 but lacks the 3′-UTR. These findings led to the conclusion that the identified clone of the cerebellar subtractive library corresponds to a part of the 3′-UTR of the rat CRHSP-24 mRNA. This clone seemed interesting for further expression analysis as the corresponding protein functions as nucleic acid binding protein (see Discussion, section 4.4) and the CNS expression of CRHSP-24 had not yet been examined, neither in the rat nor in other species. To confirm the differential expression of this gene during development, Northern hybridisation of total RNA from multiple rat brain tissues at different developmental stages was performed with the isolated CRHSP-24-fragment as cRNA probe (*fig. 3-9*).

In summary, the results showed the presence of two rCRHSP-24 transcripts in all brain regions tested, a larger transcript at about 2,9 kb and a smaller one at 1,7 kb. The larger transcript was strongly expressed at late embryonic stages in all four brain regions analysed and showed a clear downregulation in cerebral cortex, hippocampus and cerebellum with only weak expression in the adult. In cerebral cortex as well as in hippocampus, the peak expression was before birth (E18 was the earliest stage examined) and then declined until P10. Moderate transcript levels were still expressed in cerebral cortex at P10, whereas hippocampal expression at P10 had already reached the low levels seen in the adult. In cerebellum, downregulation occurred more slowly: expression was maximal at P0, declined to moderate levels at P10 which stayed until P35, and then further decreased in the adult. In brainstem, transcript expression was strong at E18, increased until P10 and was downregulated at P35.

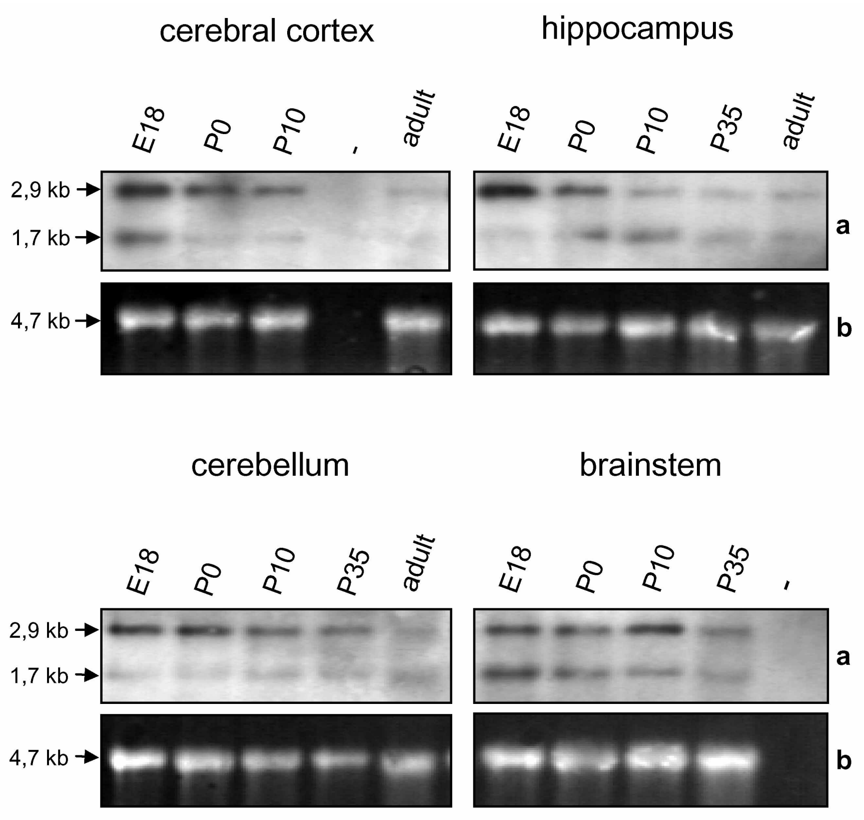

Fig. 3-9: **Northern analysis of rat CRHSP-24 mRNA in multiple brain tissues during development.** The membrane containing 5 µg total RNA of multiple brain tissues from different developmental stages (embryonic day 18, postnatal day 0, 10, 35 and adult) was hybridised with 300 ng/ml of DIG-labelled rCRHSP-24 cRNA probe (top panels, a). Exposure time of the X-ray film was 1h. Rat brain tissues from which RNA was isolated and their developmental stages are indicated at the top of each figure. No preparations of total RNA from P35-cortex and adult brainstem were available. As a control for equal loading of total RNA on each lane, a UV-photograph of the 28S-rRNA on the membrane after overnight blotting is shown (bottom panels, b). The larger of the two rCRHSP-24 transcripts (2,9 kb, upper panels) was strongly downregulated in cerebral cortex and hippocampus early in postnatal development. In cerebellum, downregulation occurred more slowly: expression was maximal at P0, declined to moderate levels at P10 which stayed until P35, and then further decreased in the adult. In brainstem, peak expression occurred at P10 and was downregulated at P35. In the adult, weak transcript levels were still present in all brain regions tested.

In situ-hybridisation was performed with the rCRHSP-24 cRNA probe on rat sagittal brain sections of different developmental stages to examine the distinct expression pattern of rCRHSP-24 during neuronal differentiation in the CNS. Unfortunately, it was not possible to obtain satisfactory in situ-hybridisation data for this clone although many different protocols were tried. Even with a rCRHSP-24-riboprobe which was newly generated based on the published cds of the rCRHSP-24 gene and which was non-overlapping with the originally used probe, the efforts to obtain specific hybridisation signals remained without success.

3.2.1.2 Isolation and characterization of the rat gene for ubiquitin-conjugating enzyme E2 variant MMS2

A different subtracted clone (clone 14) displayed a significant alignment with the mouse gene for ubiquitin-conjugating enzyme-like protein MMS2 (accession number AK019486; 350 identities of 358, 97% identity, Score: 646 bits and e-value: 0 with BlastN;), which is a highly conserved protein even in non-vertebrates, e.g. yeast. In yeast it was first isolated and discovered as a protein which is highly similar to a family of ubiquitin-conjugating enzymes (E2-enzymes), but which lacks the essential cysteine residue at the active site and therefore is not functional in ubiquitin-conjugation. The isolated 373 bp cDNA fragment of clone 14 (deposited in DDBJ/EMBL/GenBank databases under accession number AJ 515244) was highly similar to the 5'-end of the cds for mouse MMS2, the total length of which is 438 bp. Searching for overlapping rat ESTs delivered a sequence with the accession number BG 664464 (Score: 673 bits, e-value: 0) which contained the complete cds for the rat homologue of mouse ubc-like protein MMS2. The open reading frame (orf) consisted of 145 aa and the predicted molecular weight for rat MMS2 protein was 16 kDa. Another rat EST-sequence (accession number BI 295928) was found to overlap with BG 664464 (97% identity) at the 3'-end. A search in the rat HTGS-database yielded a genomic sequence (accession number AC114032) that correctly overlapped with the originally isolated clone and also with both EST sequences described above. The overlapping sequences were interrupted by two sequence stretches, indicating that the rat MMS2 gene contained at least two introns. AC114032 further supplied the 3´-UTR of the gene, which was highly similar to the mouse homologue (AK019486). By compilation of the initially isolated clone from the cerebellar subtractive cDNA library and the sequence fragments from AC114032, a 3248 bp sequence was obtained which is shown in *fig. 3-10.* This sequence displayed 91% identity to the mouse homologue in the coding region and between 80 % and 91% identity in the 3´-UTR. The deduced amino acid sequence shared 99% identity with mouse MMS2 as well as with the human homologue (human ubiquitin-conjugating enzyme E2 variant 2, Ube2v2, or human MMS2) and was 50% identical to yeast MMS2. Northern hybridisation results indicated that this 3248 bp DNA represented the full length transcript, as an approx. 3 kb-transcript was detected in addition to a major 1,7 kb-transcript (see also section 3.2.1.3). This is further supported by the sequence length of the mouse cDNA for MMS2 (3272 bp), which was isolated from a full-length enriched library and correlates well in size to the rat sequence described above. Based on the striking sequence similarity, the 3248 bp DNA fragment was thus designated as rat MMS2 cDNA and deposited in the DDBJ/EMBL/GenBank databases (accession number: BN000090).

```
                            M  A  V   S  T  G  V   K  V  P   R  N  F
   1 ACGCGGGGGA GAAGGAGAAG ATGGCGGTGT CCACAGGAGT TAAAGTTCCT CGTAATTTTC

     R  L  L  E   E  L  E   E  G  Q   K  G  V  G   D  G  T   V  S  W
  61 GCTTGTTGGA AGAACTTGAA GAAGGACAAA AAGGAGTAGG TGATGGTACG GTTAGCTGGG

     G  L  E  D   D  E  D   M  T  L   T  R  W  T   G  M  I   I  G  P
 121 GCCTTGAAGA TGATGAAGAC ATGACACTTA CAAGGTGGAC AGGCATGATT ATTGGGCCAC

     P  R  T  N   Y  E  N   R  I  Y   S  L  K  V   E  C  G   S  K  Y
 181 CAAGGACAAA CTATGAAAAC AGAATATACA GCCTGAAAGT AGAATGCGGA TCTAAATACC

     P  E  A  P   P  S  V   R  F  V   T  K  I  N   M  N  G   I  N  N
 241 CAGAAGCACC TCCATCCGTT AGATTTGTAA CAAAGATTAA TATGAATGGG ATCAATAATT

     S  S  G  M   V  D  A   R  S  I   P  V  L  A   K  W  Q   N  S  Y
 301 CCAGTGGAAT GGTGGATGCA CGGAGCATAC CAGTATTAGC AAAATGGCAA AATTCCTATA

     S  I  K  V   V  L  Q   E  L  R   R  L  M  M   S  K  E   N  M  K
 361 GCATTAAAGT TGTACTTCAA GAGCTAAGAC GTCTTATGAT GTCCAAAGAA AACATGAAGC

     L  P  Q  P   P  E  G   Q  T  Y   N  N
 421 TTCCACAGCC ACCAGAAGGA CAGACATACA ACAACTAATT TTAGTGGATC TCAAACTTGT
 481 CTTAAATCAA CAACCTTCTA CTCATGTTAA TGTCTTGATT AAATATCACC ATGCAAAATA
 541 CCCACACATT AAGTAAAAGA ATTGCAGCTG GTAAACATGA CCTGGACAGT TGTAAGAATA
 601 TATTTAATAT ATGTACACCT ATTATGTTTT CAGGTAACAA GAGAAGTGCA GCACAATTTT
 661 TCTTTCTCTT CTTAAAAGCA CTGTCATTTA AACATGAATC TGAAGCATTC AAAATGGAAT
 721 TCAGGTTGTC AAGACAGAAG CATGGCAAAA GAGTGTCAGA TGGCCACGGG AACCTTTGTT
 781 TTTCTGAAAG TAAAGTCTCA AGAAAGAAGA ACAGAACTGG CAGGCTTTAT CCACCATATT
 841 TAGAGCTGCA GATGGAATTG TTTAAAGTAG CAGACATAAT GACTAGTCAC CAATTGTGTT
 901 AATCCTTGAA GCGGTGTGGG TGCTGACTAC TGTTAGTGTC AAACATAGGT GCAGGATGGT
 961 TTTGATACCT GTATTTATAA TAAAAAGATG TTTGGGGTTT TGAATTTCTG TTAAAAGCTG
1021 TTCTCGTTTG TTACATGTAA CAGACATGAT AAGTTGTTTA CAGTCTTTGT TTAATAAAAC
1081 ATGCTTAGAA GTTTTAAGTG AAGACAACAA AAAGGAAATA GGTGTTGGAT ATGTGATTTT
1141 GAGATGAAAG TTAGTCTTAA AATGTAAATA AAATATGGAA TGTGTCTTCA AAGACTGTGC
1201 TATTTCTGTT ACATTGATAT ATGCTATAGT AGCCATATCA AGGTAGCAAA ATTGGGGGTT
1261 ATTAAGCTAG GCATGGTAAT GGCAAAAGCA GGAAGATTTT CAGTTCCAGG CCAGCTTGGG
1321 CTGATACATA AGATTGTATG TAAAACCAAA GAAATAATGA GAGAATATCA AAGATTTTCA
1381 TTGAAATTCT ATTTTATATG CTTATGTTTG GAACTTACAG AGCATCTTTT TGACTTCTTA
1441 CATGCTCAGT TTAATTTCTT TGCACAAAGA GGTATCTCGG TTACTACATT TCAAGTTTTA
1501 TTTCATCATG GGTAATTTTA AAATTGCATT TTAGCAATTT TCATAGTTAC AGACTGCAAA
1561 TTATTCAAGT ACTCATTCAT TTTTTTCTTT ATGACTTATT TTTCTATGAA GCTTTTGGTT
1621 CATTACAGTT GACTGAATTG AAGACATTTC TTCACCTGTC TTTACATTTT TTTTTTACTT
1681 TATTTGTGTG TGTGTATGTG TGTTGTGTGT GTGCGCACAT GCACATATGT GTGTGTACAC
1741 TTGTATGAAG GTCAAAGGTT ACCTTTGGAG TTGGTTCTTG CCTTCACTTT TATATGTGTT
1801 CCTGATGTCA AACTCTGATC ACCATGTACC ATCTGTCTGA ATAAGGGAAT GAGTGCCTTC
1861 ACTGAAGAGT CATCTCATTG GTCCCTCTTC TAAGACCAAT GAAAGTTAAT TTTATCATTC
1921 CACTCCACAT TCACTTTAAC AAATATGTGT CTGAAGTTGA AAGTTCATTT TTATTCTTTT
1981 ATCATATTGA CAAAGTTTGA TTGTCAAAAT AAAAAATTAG ATGTTCAAGC TTTGCCTATA
2041 AAGAAAGTAA AGGAAAAGTA TTACTAGTAG ACTGGATTAT TTTGGTTACT TTTATAGCCA
2101 AGACAGGTTT CTTTAAACAT ACTTGGCATG TTTTGATTAA GCTGTTTAAA AATTAACCTT
2161 TTTTTAGTTA GCATTTCATT AATTAAACAC TAGACATTTT GTCTAAACAT TATCATCACT
2221 TTTGTATTTT GAAACAGTAT CACCTATGAT AGCCTCAAAC TTGCAGAATT ACTTCTGTCT
2281 CAACCTCTCA AGTACTGGTT CTGGTGTACA CTGCAATGTC CAGAATAGAT GGACTCATTT
2341 TAAATGAACT AGTTAATTAT CAGCGAGGTT TCTTTATTCT TTGTGTGTGT GTGTGTGTGT
2401 GTAGGTGGGG AGGAGTAGTG GTGTATACAG AAGCCTTAGG TTGACGTCTG GAGTCTCCTT
2461 TAATTGCTCT TTCACTTTAT ATAGTGTGAG ACAGGGTTTC TAAATGAAGC TCACTGATGC
2521 GGCTAGACTT GCTAATCAGC TTGCTACCAA GATAGATCCC CTGCCTCTGC TTTCCAAGGC
2581 CGGAATTACA GCCTCAACTG TTATTTTATG TGGGTTCTAG GGATCTGATA TCCGACGAGC
2641 ATTTTCGACC ATTGAGCCAT CTCAGTCCCT CAAAAATGTT CTAAGTATTT AAAAATCACA
2701 CAGAATGTAC ACATTTTCCA TGCGTTAATT ATTAAAGTAG ACTTAAACAT TATACTTAAT
2761 ATTTAATGGT TAAAGATTAC ATTGAATTGC GATACTATAG AATCATTATC AGTCTGTTTT
2821 CTGCCTTGAA AATGTGTCTT GTGATACTTG GAAACTAGAA AGTATTGGGA TGCTTTTGAT
2881 AGGCTCTGAT AGAATCTCGT ACTTTGGTCT CATGTTGTCT TTGTGTATGC TACCCAAGGC
2941 TGTTGTCAGA CAGAGTCCTA CCTATTTAAA AAAAAGGGGG ATTGTTGATT TTAGCAGAAA
3001 TCTACGAGTC AGTTAAAAGA TTTCCAAAGC AAAATCCATT TTGCATGTAG CAATTATATA
3061 TGATAATCTA AGATATATTA ATTCATGAAA TTTTAGAGTC TTATAATTAT AAGACAGAAG
3121 TTTAAAAGGA AATTTTCCTT TGTAAAGTGG CTAGGGTTGC AGGAATGTTG TGGTATAGAC
3181 TAGAAAGTTG CCTTAGAAAT TTGTTTCAAG AGATTTTTAA GGATGACCCT TGATGCCTGT
3241 ATGTTAAT
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Fig. 3-10: The nucleotide sequence of rat MMS2 cDNA.
The sequence was constructed from the subtracted cDNA clone 14 (the first 373 bp, deposited in the DDBJ/EMBL/GenBank databases under accession number AJ515244) and an overlapping rat genomic-sequence from the HTGS database (AC114032). Coding sequences were identified by homology to rat ESTs (BG664464 and BI295928). The numbers in the left column refer to the nucleotide positions. The predicted amino acid sequence of rat MMS2 polypeptide (145 aa) is shown above the corresponding nucleotide sequence. The sequence of rat MMS2 cDNA has been deposited in the DDBJ/EMBL/GenBank databases (accession number: BN000090). A potential phosphorylation site for cAMP-dependent and Ca+/ calmodulin-dependent protein kinases is highlighted with a box.

3.2.1.3 Northern analysis of rMMS2 transcript in brain from late embryonic development until adulthood

In order to clarify whether rMMS2 might possibly be involved in the establishment of neuronal connectivity in the developing CNS, the first aim was to elucidate its expression pattern in brain during postnatal development. Therefore, the developmental regulation of rat MMS2 expression was studied by Northern hybridisation analysis on several brain regions at different developmental stages *(fig. 3-11)*. The cRNA-probe for rMMS2, which was made from the originally identified cDNA fragment and is specific to a large part of the cds, hybridised with a major transcript at 1,7 kb in all brain regions tested. A minor transcript, between 2,7 and 3,7 kb in size, was also observed, but, at the probe concentrations used, the signal for this larger mRNA was very weak and was visible only in cerebral cortex and hippocampus at E18, independent of the exposure time (not shown). The 1,7 kb transcript is likely to be generated by alternative splicing as the gene for rMMS2 consists of several exons (see section 3.2.1.2) or, alternatively, might arise by usage of an alternative polyadenylation site. The results revealed a high expression of rat MMS2-mRNA in cerebral cortex, hippocampus and cerebellum at late embryonic development (E18) and a strong downregulation at birth in hippocampus and at P10 in cerebral cortex and cerebellum. The transcript was further downregulated at P35 in hippocampus and cerebellum and was absent in the adult. In brainstem, the signal was weaker and remained constant until P10 but was absent at P35. After overnight exposure of the X-ray film to the chemiluminescent signal, low transcript levels could also be detected in the hippocampus, but not in cerebral cortex, cerebellum and brainstem.

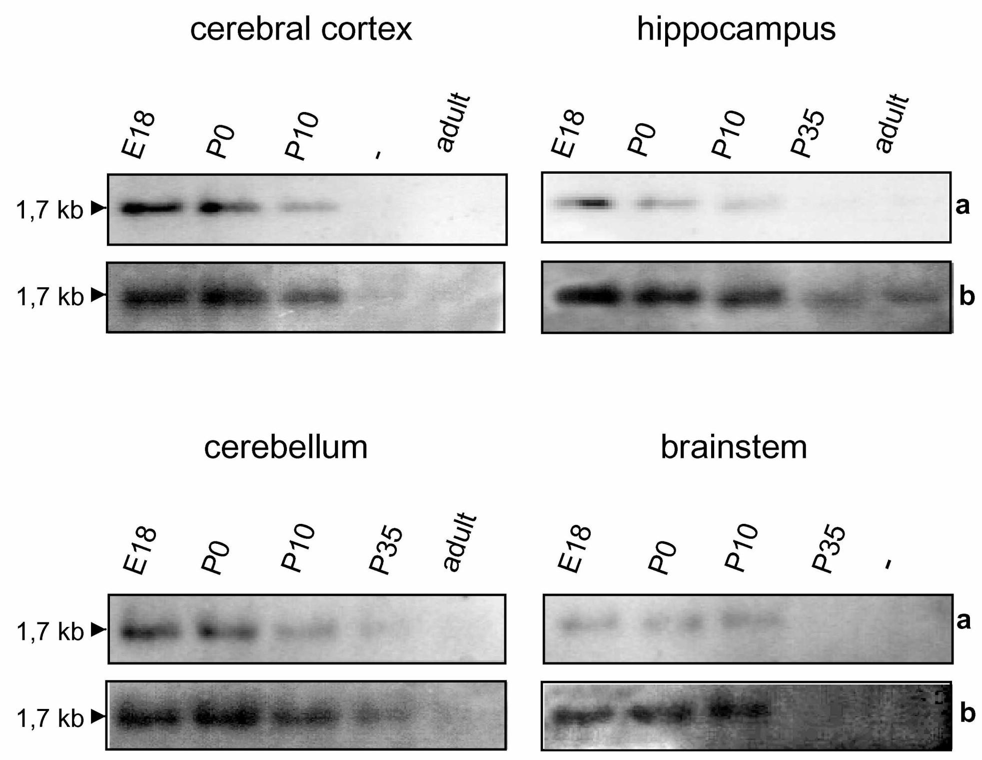

Fig. 3-11: Northern analysis of rat MMS2 mRNA in multiple brain tissues during development.
The membrane containing 5 µg of total RNA (the same that was used for hybridisation with rCRHSP-cRNA probe in fig. 3-9) was stripped as described in Materials and Methods and subsequently hybridised with 300 ng/ml of DIG-labelled rMMS2-cRNA probe. Exposure time of the X-ray film was 15 min. (upper panels, a) or overnight (lower panels, b). A control for equal loading of total RNA on each lane was shown in fig. 3-9 (lower panels, b). Rat brain tissues from which RNA was isolated and their developmental stages are indicated at the top of each figure. No preparations of total RNA from P35-cortex and adult brainstem were available. The rMMS2-probe specifically hybridised with two transcripts, a major 1,7 kb mRNA and a minor transcript of approx. 3 kb, which, at the probe concentration applied, was only weakly detectable in cerebral cortex and hippocampus at E18 (not shown). The major 1,7 kb rMMS2 transcript was strongly downregulated in cerebral cortex, hippocampus and cerebellum early in postnatal development. In brainstem, expression remained constant until P10 but was decreased at P35. In the adult rat, weak expression of rMMS2 could only be detected after overnight exposure in the hippocampus.

3.2.1.4 Transcript expression of rMMS2 in neonatal rat brain

To further gain insight into the distribution of rMMS2 transcript in rat brain around birth, in situ-hybridisation on rat sagittal brain sections was performed at developmental stages E20 and P1.

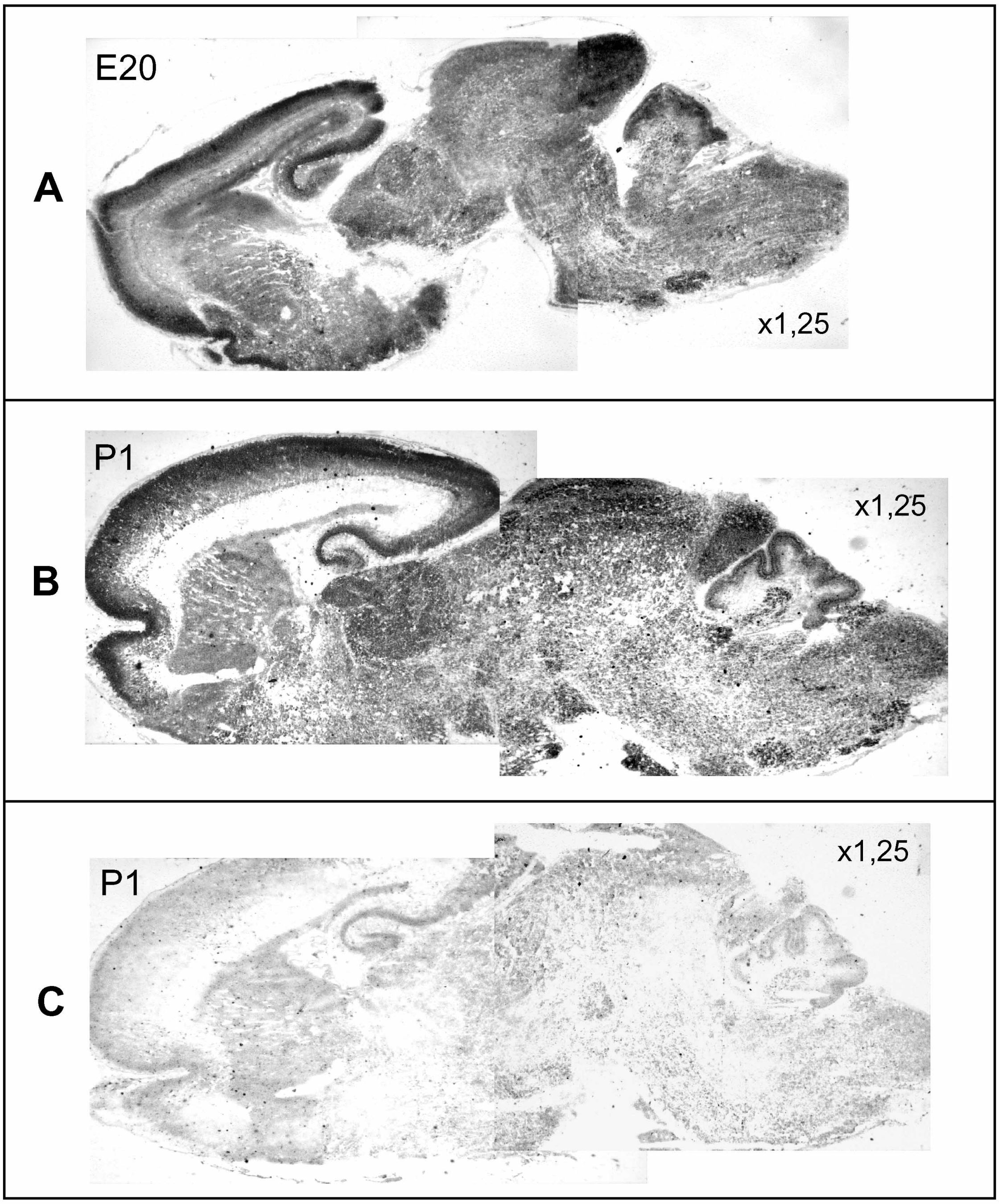

Fig. 3-12: Distribution of rMMS2 transcript in rat brain around birth.
Sagittal brain sections from rats at embryonic day 20 (A) or postnatal day 1 (B and C) were hybridised with 500 ng/ml of either antisense (A and B) or sense (C) probe for rMMS2. For each picture, two separate photographs of the forebrain and the hindbrain were combined to show the entire brain. rMMS2 mRNA is widely expressed in rat brain around birth with highest transcript levels being detected in the outer layers of the cerebral cortex, pyramidal cells of hippocampal regions CA1-CA3, thalamus (P1), superior (P1) and inferior colliculus of the tectum, cerebellar cortex, deep cerebellar nuclei and some brainstem nuclei.

In *fig. 3-12*, an overview of rMMS2 mRNA expression around birth is presented in the entire rat brain. Hybridisation of a brain section at E20 with the antisense rMMS2-cRNA probe revealed widespread expression of rMMS2, most likely in neurons (see *fig. 3-14* and 3-15 for higher amplifications). Highest levels found in the outer layers of the cerebral cortex (cortical plate), pyramidal cells of hippocampal regions CA1-CA3, thalamus, superior and inferior colliculus, cerebellum and in a few brainstem nuclei (A and B). In cerebellum, deep cerebellar nuclei were stained as well as the cortex. A control section, hybridised with a sense rMMS2-cRNA probe under same conditions, did not show any significant signal (C).

3.2.1.5 Downregulation of rMMS2 mRNA in postnatal rat brain

To confirm rMMS2 downregulation observed in Northern analysis, the spatial and temporal distribution of rMMS2-mRNA in brain during postnatal development and neuronal differentiation was further examined. rMMS2 expression was therefore studied in the CNS at different developmental stages by in situ-hybridisation *(Fig. 3-13 to Fig.3-16).* Sense hybridisations carried out under the same conditions as the antisense experiments did not display significant signals. Only at P35 and in the adult rat some background staining was detected in the cerebellum and hippocampus. These regions are known to be delicate in analysis by in situ-hybridisation as they often exhibit unspecific signals when hybridised with sense probes. The signals obtained in cerebellar granule cells and in the dentate gyrus of the hippocampus at P35 are therefore likely to be unspecific. *Fig.3-13* illustrates the transcriptional regulation of rMMS2 throughout postnatal development in cerebral cortex (A-H), hippocampus (Q-X) and cerebellum (I-P); higher power photographs are shown in *fig. 3-14* (cortex and hippocampus) and *fig. 3-15* (cerebellum). In cerebral cortex (*fig. 3-13*: A-H and *fig. 3-14*: A-C), a strong staining for rMMS2 could be observed in the outer half of the cerebral cortex, most probably corresponding to postmitotic neurons of the cortical plate (CP) and subplate. At P5, a signal was observed in two cell bands of the cortical plate: the outer band included cells near the pial surface, the inner band comprised neurons of middle cortical layers. At P10, rMMS2 expression became restricted to neurons of middle layers of the cortical plate (layers 5 and 6). At P35, the signal strongly decreased and was further diminished in the adult. In cerebellum (*fig. 3-13*: I-P and *fig. 3-15*: A-C) highest transcript levels were found at P1, in the deep nuclei and also in the cortex, where cells of the EGL (premigratory granule cells, precursors of Bergman glia and Stellate cells) and of the Purkinje cell layer (PL) expressed rMMS2. At P10, a weak staining was still observed in the EGL and in some cells of the deep nuclei. At P35 and in the adult, transcript expression was strongly reduced and remained weak in the granule cell layer and deep nuclei. In the hippocampus (*fig.3-13*: Q-X and *fig.3-14*: D-F), the signal was strongest in pyramidal cells of the CA3-region at P1, became weaker at P10 and was decreased to very low levels at P35. In the adult, rMMS2 expression was absent in the entire hippocampal formation.

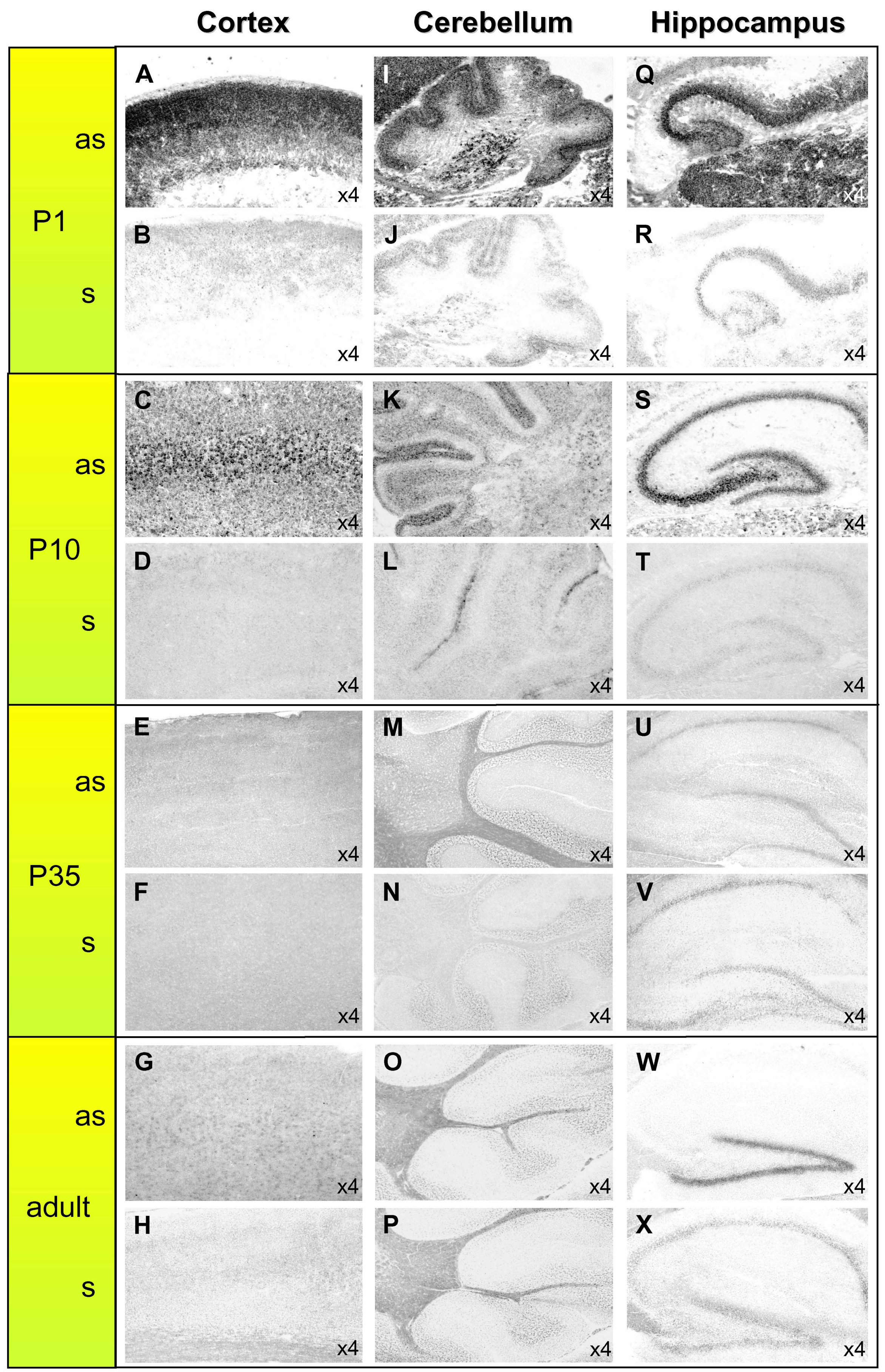
Cortex
Cerebellum
Hippocampus
P1
as
s
A
I
Q
B
J
R
P10
as
s
C
K
S
D
L
T
P35
as
s
E
M
U
F
N
V
adult
as
s
G
O
W
H
P
X
x4

Fig. 3-13: Developmental expression pattern of rMMS2 transcript in rat cerebral cortex, hippocampus and cerebellum.

Sagittal brain sections from rats of the indicated ages were hybridised with 500 ng/ml antisense or sense cRNA probe for rMMS2. Bright field photographs from cerebral cortex (A-H), hippocampus (Q-X) and cerebellum (I-P) demonstrate a strong transcriptional downregulation during early postnatal development. In cerebral cortex, a strong signal was distributed over the outer half of the cortical plate at P1 (A), whereas it was restricted to middle cortical layers at P10 (C) and expression decreased further until adulthood (E and G). In the cerebellum of a P1-rat, rMMS2 transcript levels were high in the deep nuclei and somewhat weaker in the cerebellar cortex (I). At P10, the signal was decreased in the deep cerebellar nuclei but stayed at moderate levels in the external granular layer, EGL (K). At P35 and thereafter, rMMS2 mRNA levels were hardly detectable (M and O). In hippocampus, highest transcript levels were seen in the CA-regions until P10 (Q and S). It seemed that rMMS2 was higher expressed in the CA3- than in the CA1-region. At P35, expression in the CA-region was much weaker (U) and decreased further until adulthood (W). Hybridisations performed with a sense probe for rMMS2 at the same concentration under same conditions did not show any signal until P10. However, at P35 and in the adult, a weak signal was found in the cerebellar granule cells and dentate gyrus of hippocampus similar to the signals seen with the antisense probe. These signals therefore are likely to be unspecific.

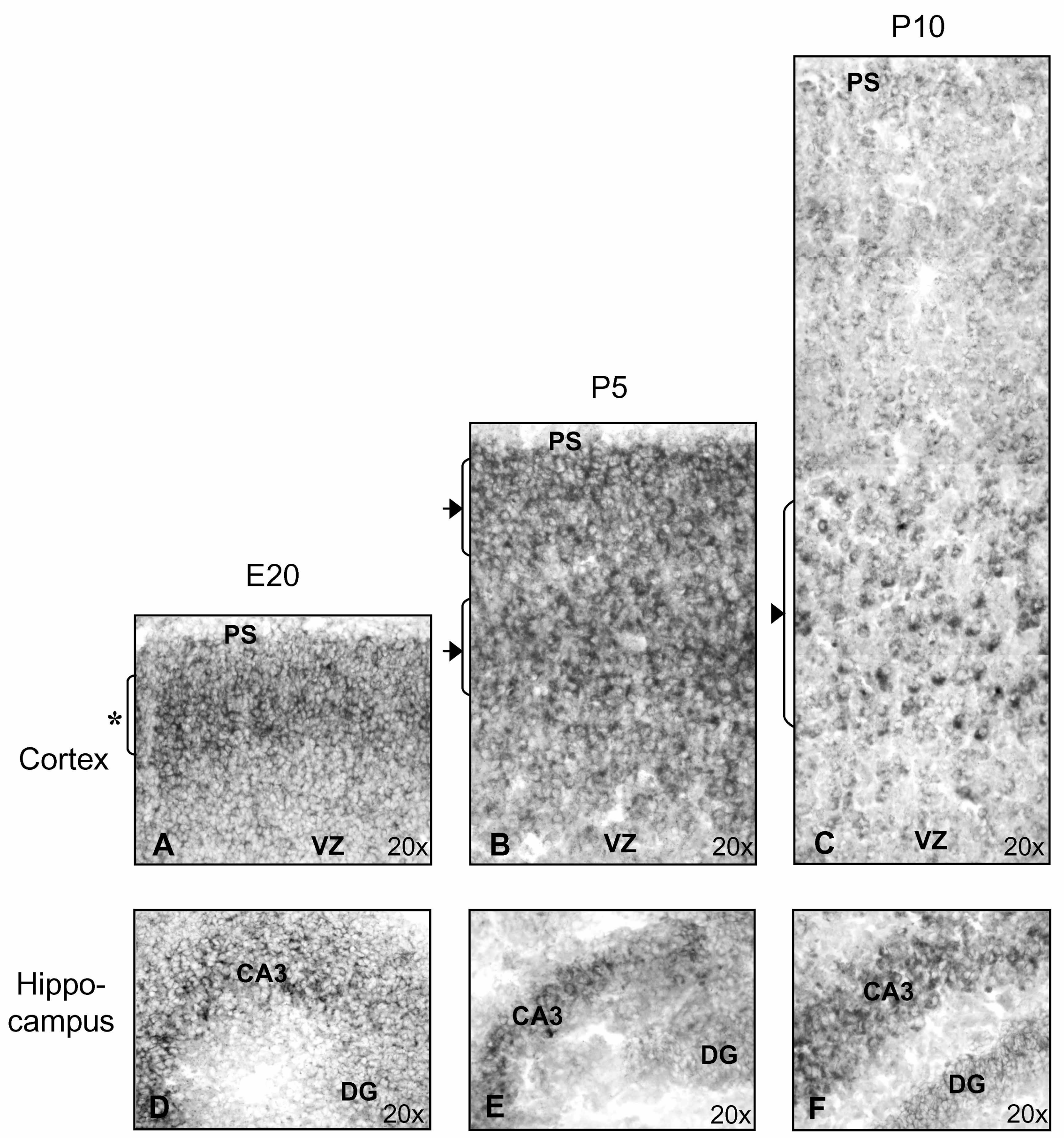

Fig. 3-14: Higher resolution of rMMS2 transcript expression patterns in rat cerebral cortex and hippocampus during postnatal development.

Transcriptional downregulation of rMMS2 during development (demonstrated in fig.3-13) is shown at a higher magnification (20x) in the cerebral cortex (A-C) and the CA3-region of the hippocampus (D-F) in rats from developmental stage E20, P5 and P10. Hybridisation conditions were the same as described for fig.3-13. At E20, neurons of superficial cortical layers were stained for MMS2-RNA (indicated by an asterisk in A). At P5, superficial and middle cortical layers were positive (indicated by arrows in B). At P10, the signal has become restricted to neurons of middle cortical layers (indicated by an arrowhead in C). In the hippocampus, CA3 pyramidal neurons exhibited a strong signal from E20 until P10, whereas the granule cells of the dentate gyrus displayed merely weak to moderate MMS2 transcript expression (D-E). PS = pial surface, VZ = ventricular zone, DG = dentate gyrus, CA3 = CA3-pyramidal cells of the hippocampus.

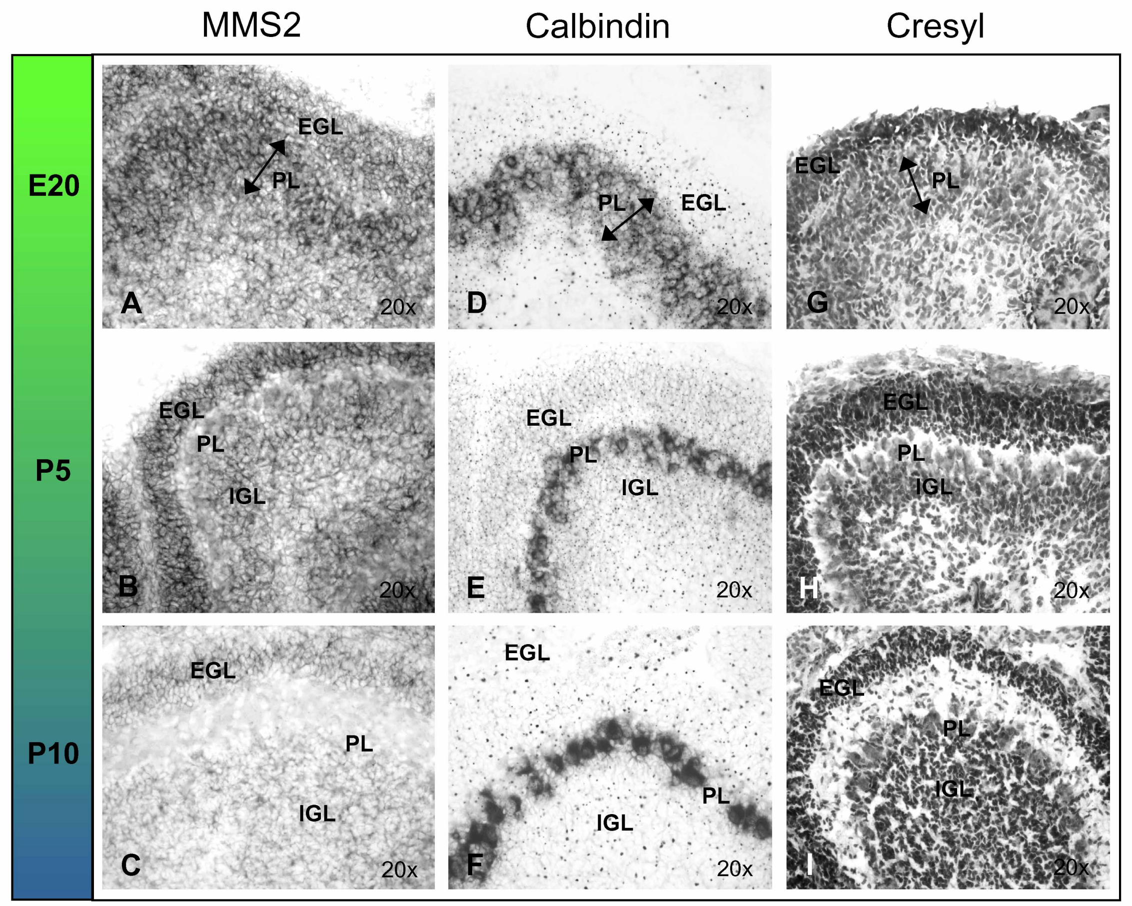

Fig. 3-15: **Higher resolution of rMMS2 transcript expression in the rat cerebellar cortex during postnatal development.**

Transcriptional downregulation of rMMS2 in the cerebellum during postnatal development (demonstrated in fig.3-13) is shown at a higher magnification (20x). Hybridisation of sagittal brain sections from rats at E20, P5 and P10 was carried out with either 500 ng/ml antisense cRNA probe for rMMS2 (A-C) or with 100 ng/ml antisense cRNA probe for Calbindin (D-F), which specifically labels Purkinje neurons in the cerebellum. In G-I, control sections were stained with cresyl violet in order to better discriminate cellular layers of the cerebellum: PL = Purkinje cell layer, EGL = External granular layer, IGL = Internal granular layer. At E20, hybridisation with the MMS2-probe (A) resulted in a strong staining of cells located in the PL and a moderate staining of cells of the EGL. Hybridisation with the Calbindin probe at E20 (D) identifies Purkinje neurons in a thick cellular band underneath the EGL, as they have not yet formed a monolayer. At P5, MMS2 transcript expression was still present in neurons of the superficial EGL but was reduced in deeper layers of the cerebellar cortex (B): in the PL, transcript expression was very weak, whereas cells in the IGL were clearly positive. At P10, MMS2 expression has decreased to low levels in the EGL and IGL and was completely absent in the PL.

Fig. 5-16 demonstrates the developmental regulation of rMMS2 transcript in the brainstem (A-F), thalamus (G-L) and tectal area of the mesencephalon (M-R). In brainstem, several nuclei (pontine nucleus, facial motor nucleus, superior and inferior olivary nuclei and a nucleus in the isthmal region of the cerebellum) were positive at P1, but rMMS2 expression was strongly downregulated at P10 and completely absent at P35. In the thalamus, a strong signal could be observed at P1 in the anterior and intermediate thalamus. This expression was strongly downregulated and hardly detectable at P10, no hybridisation was found at P35. In the tectal region, highest transcript levels were found in the superficial layer of the superior colliculus and in the inferior colliculus at P1. This signal was much reduced at P10 with only weak expression being detected in the superior colliculus and was completely absent at P35.

The detailed expression analysis by in situ-hybridisation confirmed that the rMMS2 transcript was widely expressed in neurons at prenatal and neonatal stages but became strongly downregulated in all brain areas after the first postnatal week. This time course corresponds well with the loss of the potential for axonal growth and sprouting found in many neurons.

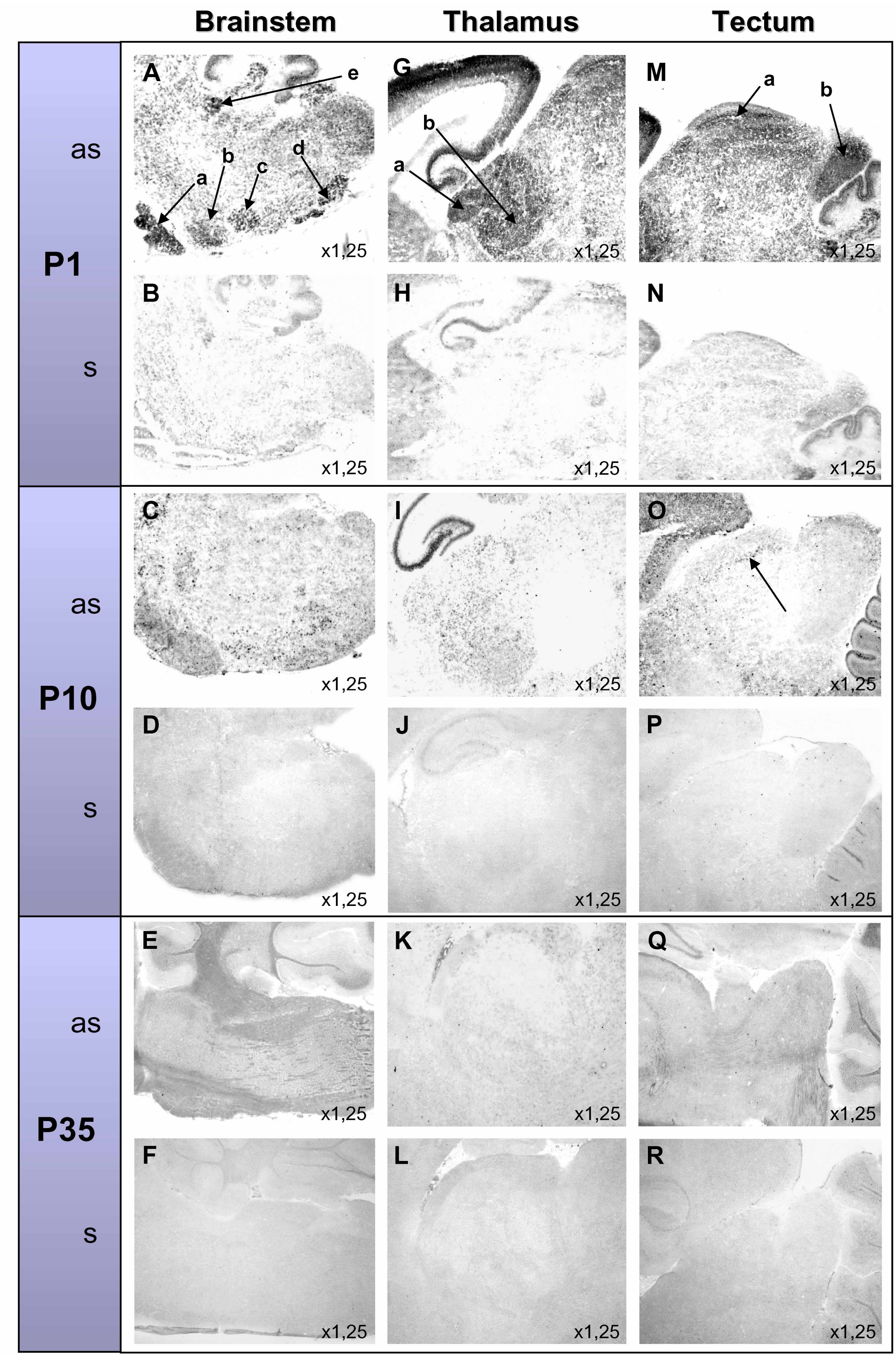
Brainstem
Thalamus
Tectum
P1
as
s
P10
as
s
P35
as
s
A
a
b
c
d
e
x1,25
G
a
b
x1,25
M
a
b
x1,25
B
x1,25
H
x1,25
N
x1,25
C
x1,25
I
x1,25
O
x1,25
D
x1,25
J
x1,25
P
x1,25
E
x1,25
K
x1,25
Q
x1,25
F
x1,25
L
x1,25
R
x1,25

Fig. 3-16: Developmental expression pattern of rMMS2 transcript in rat brainstem, thalamus and tectal region of the mesencephalon.

Sagittal brain sections from rats of the indicated ages were hybridised with 500 ng/ml antisense or sense probe for rMMS2. Bright field photographs from brainstem (A-F), thalamus (G-L) and tectal region (M-R) demonstrated a strong transcriptional downregulation during early postnatal development. In brainstem, a strong signal was present in several brainstem nuclei at P1 (A), which are indicated by arrows: high transcript levels were found especially in the pontine nuclei (a), superior olivary nucleus (b), facial motor nucleus (c), inferior olivary nucleus (d) and a nucleus in the isthmal region (e). At P10, rMMS2 transcript was still weakly expressed in certain nuclei (C), whereas it was completely downregulated at P35 (E). The anterior (a) and intermediate (b), but not the posterior thalamus displayed strong rMMS2 expression at P1 (G). At P10, a signal in the thalamus was hardly detectable (I) and was completely absent at P35 (K). In the tectal region at P1 (M), highest transcript expression was present in the superficial layer of the superior colliculus (a) and in the inferior colliculus (b) At P10, expression was weaker in the entire tectum and became restricted to a thin layer in the superior colliculus (O, arrow). At P35, it was completely downregulated (Q). Sense hybridisations, performed at the same concentration under same conditions, did not show any signal.

3.2.1.6 Differential regulation of rat CD24 during postnatal development

CD24 was yet another molecule identified by the cerebellar subtraction approach. It is a highly glycosylated protein, which belongs to the large protein family of cell adhesion molecules and is attached to the cell membrane by GPI-anchoring (Kay et al., 1991; Pirruccello and LeBien, 1986). As CD24 is differentially expressed in the developing mouse cerebral cortex (Calaora et al., 1996) and in mouse and rat cerebellum (Kuchler et al., 1989) and is downregulated in the adult brain in human, mouse and rat (Poncet et al., 1996; Shirasawa et al., 1993), examination of its developmental expression pattern was performed in the entire brain during the critical period of axonal outgrowth. Northern- and in situ-hybridisation analyses *(fig. 3-17 to 3-19)* were therefore performed using the isolated CD24 cDNA fragment as hybridisation probe. *Fig. 3-17* shows the temporal regulation of the CD24 transcript in developing rat brain by Northern hybridisation. In cerebral cortex, hippocampus and brainstem, expression was strongest at E18 and declined thereafter. At P10, expression was already very weak if detectable at all. In cerebellum, expression was moderate at E18 and stayed until P10 but was absent at P35. In the adult, no transcript levels could be detected in neither of the tested samples, even after overnight exposure.

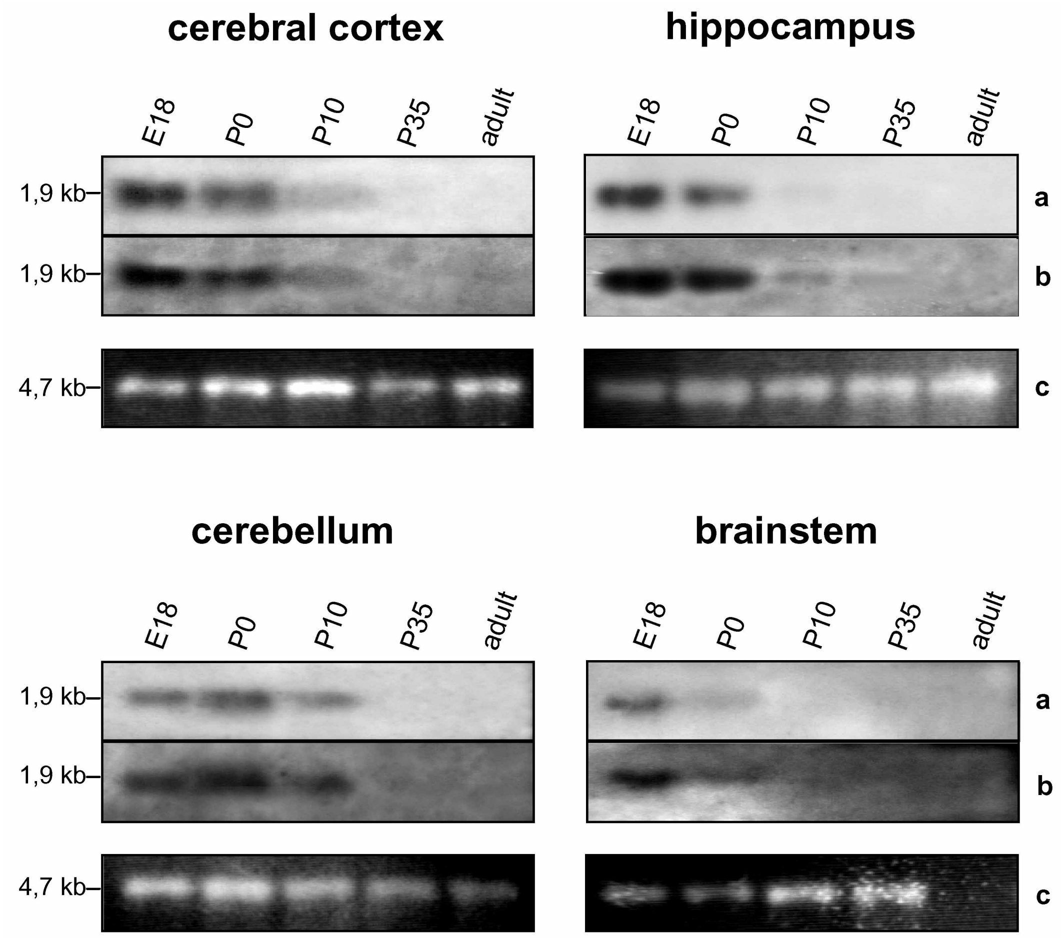

Fig. 3-17: Northern analysis of rat CD24 mRNA in multiple brain tissues during development.

The membrane containing 1 µg of total RNA was hybridised with 120 ng/ml of DIG-labelled CD24 cRNA probe. Exposure time of the X-ray film was 2 hours (upper panels, a) or overnight (middle panels, b). As a control for equal loading of total RNA on each lane, a photograph of the ethidium bromide-stained 28S rRNA band is shown (lower panels, c). Rat brain tissues from which RNA was isolated and their developmental stages are indicated at the top of each figure. The 1,9 kb CD24 transcript was strongly downregulated in cerebral cortex, hippocampus and brainstem in the first postnatal week. In cerebellum, transcript levels stayed until P10 but were absent at P35 and in the adult. After overnight exposure, slight expression could be detected in hippocampus at P10. Yet, in the adult, no expression was apparent in all brain regions tested.

In situ-hybridisation was carried out in order to further study the spatial and temporal distribution of CD24 transcript in developing rat brain *(fig. 3-18 and 3-19)*. In *fig. 3-18*, CD24 mRNA expression is presented on sagittal rat brain sections from developmental stage E20. Strong expression was found in outer layers of the cerebral cortex, in hippocampus, striatum, thalamus, superficial layers of colliculus superior, colliculus inferior, cerebellar cortex and deep nuclei and some brainstem nuclei. No signal was obtained with the CD24 sense probe.

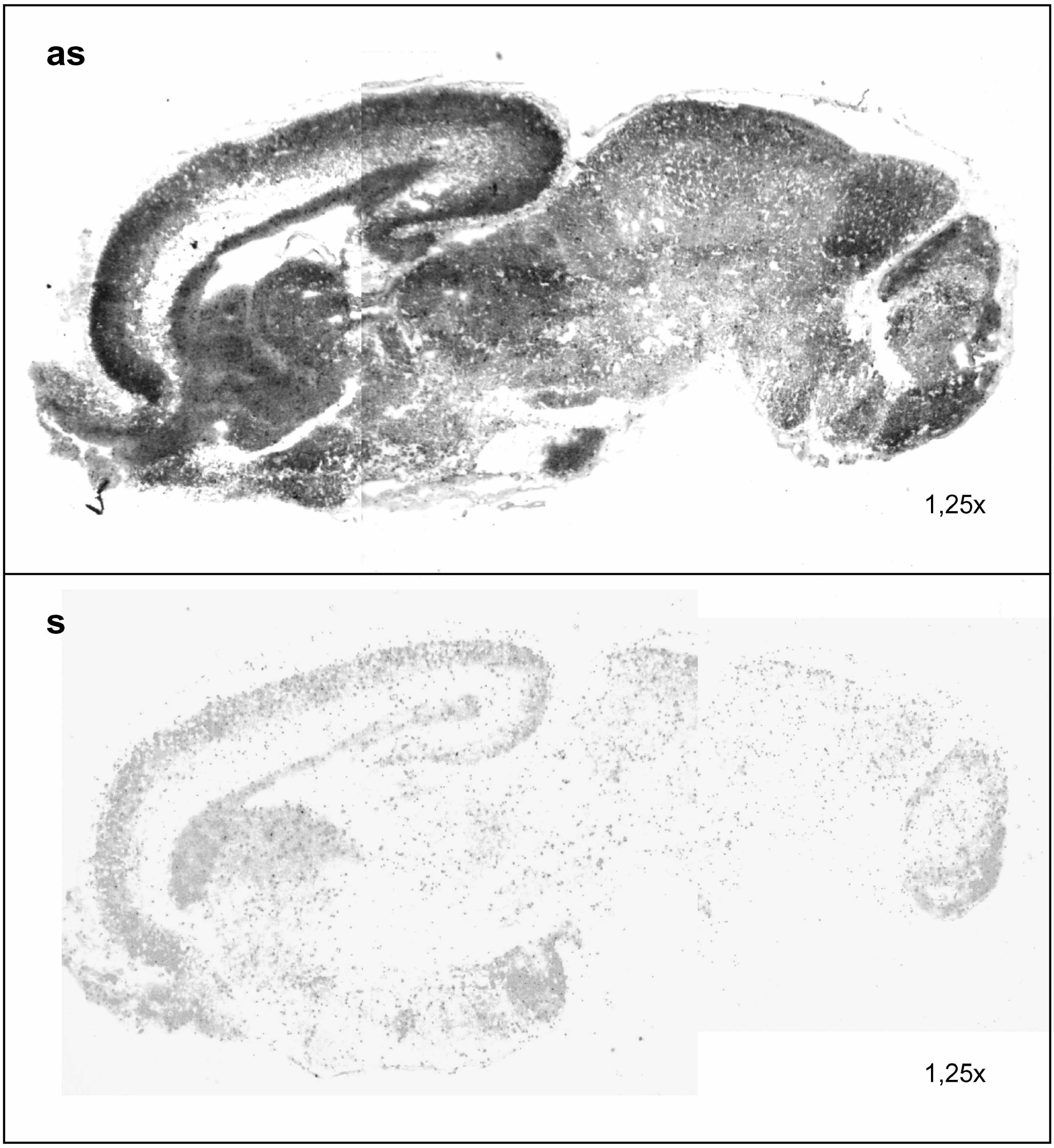

Fig. 3-18: Distribution of CD24 transcript in rat brain at E20.
Sagittal brain sections from rats of developmental stage E20 were hybridised with 500 ng/ml antisense (upper panel) or sense probe (lower panel) for rat CD24. High transcript levels were present in the outer layers of the cerebral cortex, in the striatum, thalamus, hippocampus, superficial layers of colliculus superior, colliculus inferior, cerebellar cortex and deep nuclei and some brainstem nuclei (upper panel). Hybridisation with the sense probe did not display any signal (lower panel).

The widespread expression described for E20 was strongly reduced during CNS maturation with the CD24 transcript being completely downregulated in most regions at P10, except in the cerebral cortex and cerebellum: a shift occurred in cerebral cortex from a strong signal in the outer layers at E20 to a weaker signal in the deeper layers at P10, which was absent in the adult, similar to what was described in the mouse (Calaora et al., 1996, data not shown). In the cerebellum, CD24 transcript is differentially expressed during postnatal development, which is illustrated in *fig. 3-19.* At E20 (A and B), high transcript expression was present in the deep cerebellar nuclei and in the Purkinje cell layer of the cerebellar cortex. In addition, very strong expression was observed in a brainstem nucleus in the isthmal region of the cerebellum. At P4, deep cerebellar neurons did not express CD24 transcript any more and expression in the Purkinje cell layer was much weaker and was hard to distinguish (D and E). Instead, cells of the EGL were now stained. This expression pattern remained fairly constant until P10 (G and H) but was somewhat weaker. In the adult (J and K), although CD24 expression was further downregulated, low expression was maintained in Purkinje neurons.

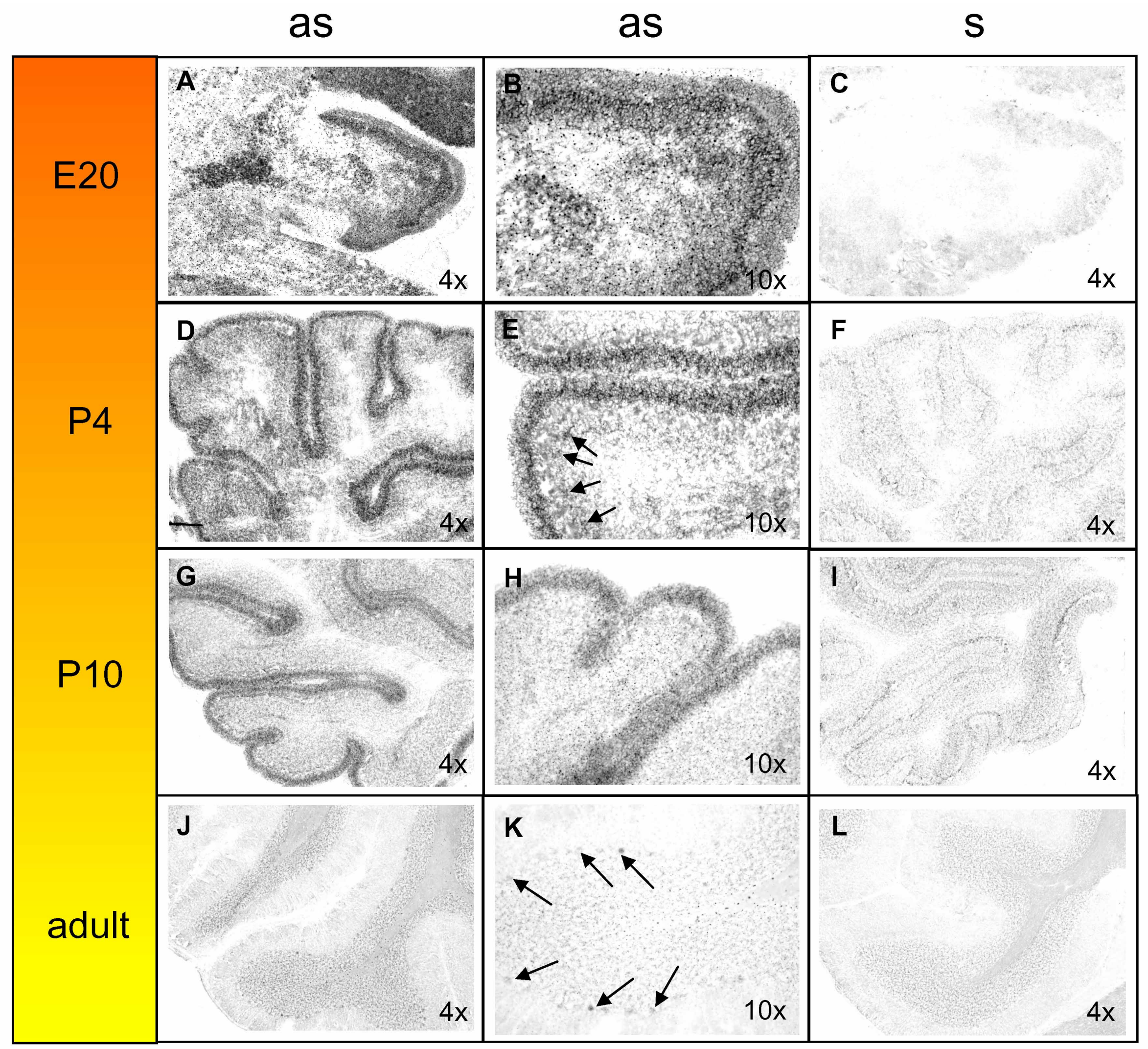

Fig. 3-19: **Expression pattern of rat CD24 mRNA in cerebellum during development.**

Sagittal brain sections from rats of the indicated ages were hybridised with 500 ng/ml antisense (left and middle panels) or sense probe (right panels) for rat CD24. Bright field photographs illustrate the developmental regulation of the CD24 transcript in cerebellum beginning at E20 until adulthood. Photographs of the middle panels show a higher magnification of those of the left panels. Highest expression was found at E20 in Purkinje cells of the cerebellar cortex and in deep cerebellar nuclei (A and B). Strong expression could also be detected in a nucleus of the isthmal region of the cerebellum (A). At P4 (D and E), expression in the deep cerebellar nuclei was not detectable anymore and had decreased to low levels in the Purkinje cell layer (arrows in E). In contrast, cells of the EGL were now positive. This pattern remained fairly constant at P10 but was somewhat weaker (G and H). In the adult (J and K), low transcript levels were still present in Purkinje cells (arrows in K), but expression was absent in the molecular layer. Sense hybridisations (right panels) did not display any signal.

3.2.1.7 Analysis of further clones of the cerebellar subtraction

In addition to the above-described molecules, further subtracted clones were examined by Northern- and in situ-hybridisation analysis. For varying reasons, these analyses were not continued. This is detailed in *table 3-2*, which presents a list of such molecules together with the performed analyses and the obtained results. On the right side, the reasons for not continuing the analysis are indicated.

Name of molecule	**Performed analysis**	**Results of analysis / Reasons for stopping the analysis**
Neuronal surface glycoprotein MRC OX-2	NB and isH	Transcript expression peaked early postnatally, declined until P10 and was further downregulated until P35 in cortex, cerebellum, hippocampus and brainstem. In hippocampus, stronger expression was observed in the CA-region than in the dentate gyrus at P4 and P10. IsH confirmed the expression pattern that was previously described in cerebellum during postnatal development. Background staining with the sense-probe.
EST-clone nr. 12	isH	Background staining with the sense-probe and a strong hybridisation gradient on the slide
Unknown clone nr. 13	isH	Only very weak and unspecific staining
GPI transamidase:	isH	Damage of brain slices and unspecifity
EST-clone nr. 20	isH	Transcript was specifically expressed in cerebellum, brainstem and cortex, but expression seemed not to be regulated in brain during postnatal development.
Ras-GAP	isH	Background staining with the sense-probe and unspecific staining with the antisense-probe
Snurportin-1	isH	Unspecifity
TAR DNA-binding protein TDP-43	isH	Unspecifity
Unknown clone nr. 29	isH	No signal
EST-clone nr. 35:	isH	Strong, unspecific signal with the sense-probe
SCG-10 and Stathmin	isH	Both probes confirmed the expression patterns that were previously described in developing rat brain. Results are already published.

Table 3-2: Expression analysis and evaluation of further clones enriched by the cerebellar subtraction approach.

This table presents a list of subtracted clones from E18-cerebellum that were examined by Northern- and in situ-hybridisation, but for which these analyses were not included in the Results section. Results of the expression studies and various reasons why these molecules were not further investigated are given in the right column.

3.2.2 Suppression Subtractive Hybridisation with rat entorhinal cortex at two developmental stages

In an attempt to identify genes implicated in hippocampal development and establishment of the perforant path, a similar subtraction approach as performed in rat cerebellum was applied for rat entorhinal cortex at two developmental stages. Comparable to the age-dependent decrease in axon growth in rat cerebellum, axonal outgrowth and regeneration is lost in the early postnatal period in the entorhinal cortex. This was studied *in vitro* in rat and mouse by diverse groups, who demonstrated that maturing entorhinal fibers lose their ability to grow towards the dentate gyrus and CA-region of the hippocampus in the second postnatal week (Li et al., 1995; Prang et al., 2001; Woodhams et al., 1993). Based on these studies, the following developmental stages were determined for the entorhinal subtraction approach: postnatal day 0 (P0) as the axon-elongating stage and P10 as the time point, when axonal outgrowth is considerably diminished. By subtraction of EC-P10 from EC-P0 transcripts, genes that are stronger expressed at birth but which are downregulated at P10 were enriched in the generated subtractive library. 45 subtracted clones were randomly chosen for sequence analysis. By comparison with the databases, 13 clones were found to be identical to known rat genes or highly similar to homologues in other species. 7 sequenced cDNA fragments were completely unknown and 11 clones corresponded to rat EST sequences. Four sequences were found twice. Remaining clones (10) didn't contain a specific insert or produced defective sequence results. Proteins encoded by known genes were classified into the same groups of proteins like in the cerebellar subtraction: (1) transcription factors and other nuclear proteins, (2) cytoskeletal proteins, (3) signalling molecules, (4) enzymes, metabolic proteins and transporters, (5) cell surface molecules and (6) growth associated proteins. The classification is shown in *table 3-3*. A few of the subtracted genes are already known to be downregulated during brain development on either transcript or protein level, which is indicated by an asterisk (*) in *table 3-3*.

The obtained sequences confirm that the generated entorhinal subtractive library may serve as a promising tool to identify genes which are strongly expressed in differentiating neurons of the CNS during formation of axons, but which are downregulated later in development. The G-protein coupled receptor SREB-2, 5 EST-clones and two unknown genes, which were isolated by the subtraction approach, were further analysed by Northern- and in situ-hybridisation. For several reasons, which were similar to those of the cerebellar subtraction approach (see *table 3-2,* section 3.2.1.7*)* and which are indicated in the following résumé, these studies did not lead to consistent results so far.

Classification groups	*Protein products of identified genes*
transcription factors and other nuclear proteins	human transcriptional activator hBRM rat nuclear transport factor-2, NTF-2 rat RNA-binding polypeptide RA301
cytoskeletal proteins	rat α-tubulin rat cytoplasmic form of χ-actin
signalling molecules	mouse arsenite-inducible RNA-associated protein AIRAP rat hfb2 protein (Bdm2) *
enzymes, metabolic proteins and transporters	rat tricarboxylate carrier-like protein human protein kinase WNK3 rat syntaxin 7
cell surface molecules	rat G-protein coupled receptor SREB2
growth associated proteins	rat GAP-43 * rat stathmin *

Table 3-3: Classification of protein products of genes, enriched in a subtractive library of rat entorhinal cortex at P0.

Specified in this list are all clones, which were identical to known rat genes or displayed strong similarity to homologous genes in mouse or human (13 of 45 sequenced clones). Proteins that were known to be downregulated during brain development either on transcript or protein level are indicated by an asterisk (*).

3.2.3 Short Résumé

The Subtractive Suppression Hybridisation approaches resulted in the enrichment of multiple rat cDNAs and allowed the identification and characterisation of a novel rat gene, rMMS2, which is developmentally regulated in rat brain. Expression patterns of further subtracted genes were analysed by Northern blotting and in situ-hybridisation studies. As was demonstrated for rMMS2, the transcripts of two more clones identified in the cerebellar subtraction assay, CRHSP-24 and CD24, were widely distributed in neonatal brain during axon outgrowth but were extensively downregulated during postnatal development and were hardly expressed in the adult. These three proteins were therefore good candidate molecules, which might play a role in neuronal differentiation. Other clones from both subtraction approaches were examined as well, some of which indicated a higher transcript expression around birth compared to later stages of development, e.g. MRC OX-2. Nevertheless, various difficulties impeded the accomplishment of these expression studies and prevented an efficient screening of the generated subtraction libraries: first, brain slices from young rats (of embryonic or early postnatal age) suffered much stronger from the long in situ-hybridisation procedure than more mature rat brains and were sometimes severely disrupted. To overcome this problem, experiments were similarly performed with perfused animals in order to work with tissue that was more robust. Yet, as sensitivity was much decreased under these conditions and experiments with many subtracted molecules resulted in only weak staining, in situ-hybridisation analyses were carried on with postfixed tissue as before. Furthermore, control experiments with corresponding sense-probes often resulted in background staining, therefore preventing a correct interpretation of the antisense-signals. Other molecules seemed not to be suitable for examination by Northern blotting or in situ-hybridisation and produced no or only unspecific signals. Finally, expression of genes like SCG-10 or stathmin were only used as positive candidates for downregulation during development but were not further examined as their expression patterns were previously studied and these results are already published.

4 Discussion

In the present studies, it was searched for genes which are highly expressed in CNS neurons during axonal outgrowth and which are significantly downregulated in these neurons during postnatal maturation when the growth-potential progressively declines. Genes associated with axon growth during development might also be important for determining the regenerative potential of neurons after nerve injury.

The idea that genes, transiently expressed during development and associated with developmental axonal outgrowth, might be re-induced after a nerve lesion and thus might contribute to axonal regeneration is supported by several studies. In the PNS, neurotrophins like NGF, NT-3, NT-4/5 and BDNF are good examples as they are expressed both, during neuronal differentiation when neurotrophic factors are crucially required for growth of peripheral nerves and development of dendritic arbors, and after peripheral nerve lesion, thus contributing to axonal regeneration in the PNS (reviewed by Bibel and Barde, 2000; Cai et al., 1999; Goldberg et al., 2002a; Markus et al., 2002). There are also candidate molecules in the CNS, whose expression is similarly regulated during formation of neuronal circuits during development and after lesion in the adult CNS. The growth-associated proteins GAP-43 and Cap-23, for example, which are expressed in growth cones of axon-extending CNS neurons and are downregulated during postnatal development in many brain regions, are re-expressed in some neurons after lesion in which they are associated with regenerative sprouting (Aigner et al., 1995; reviewed by Benowitz and Routtenberg, 1997; Bomze et al., 2001; Frey et al., 2000; Skene and Willard, 1981b). Moreover, the two cell adhesion molecules L1 and polysialylated neural cell adhesion molecule (PSA-NCAM) are developmentally regulated, promote neurite elongation and are re-induced in certain lesioned CNS neurons, which is associated with regenerating and / or sprouting axons (reviewed by Aubert et al., 1995). Furthermore, rho-GTPases are known to play a role in signal transduction of growth-controlling molecules during development and were shown to be involved in transducing inhibitory signals from myelin components in the mature CNS, thereby preventing severed fiber tracts from regenerating (Lehmann et al., 1999; reviewed by Patel and Van Vactor, 2002). Cyclic nucleotides such as cAMP and cGMP similarly exert a growth-promoting function during development and regeneration (Cai et al., 1999; Cui et al., 2003; Mizuhashi et al., 2001; Neumann et al., 2002; Qiu et al., 2002; Song et al., 1997).

These findings suggest that at least some aspects of axon pathway formation are similarly controlled during neuronal development and regeneration, which emphasizes the importance of searching for growth-associated genes during the axon-extending period of developing CNS neurons. A better understanding of developmental events may permit to address more accurately the challenge of axonal re-growth in the adult mammalian CNS.

Previously, organotypic slice cultures from cerebellum or entorhinal cortex and hippocampus had been established in the mouse and rat, which allow to extensively study the formation of neuronal connections in the CNS *in vitro* (Dusart et al., 1997; Li et al., 1995; Prang et al., 2001; Woodhams et al., 1993). Moreover, this culture model can serve well for investigations of the regenerative capability of Purkinje neurons and entorhinal pyramidal cells as both projections show a similar maturation-dependent growth-behaviour as *in vivo*. For both neuronal populations, the period of extensive axon growth as well as the time when the growth potential declines rapidly was determined (Dusart et al., 1997; Li et al., 1995; Prang et al., 2001; Woodhams et al., 1993). This culture model thus would allow the examination of candidate molecules concerning their association with axon growth or regeneration. Purkinje neurons and entorhinal pyramidal cells, respectively, were therefore chosen for analysis of their gene expression patterns at the time point of maximal axonal outgrowth during development. Two different approaches were used in order to identify genes highly expressed in these two neuronal populations during early differentiation processes. The first approach, degenerate PCR with oligonucleotides specific for transcription factor classes, focused on the identification of molecules with a regulatory function that might control the expression of several axon growth-associated downstream target genes. The second approach, subtractive suppression hybridisation (SSH), though considering various molecule classes, confined the search on those genes that were downregulated during neuronal maturation.

4.1 Degenerate PCR-approach

Degenerate PCR with primers for DNA-binding domains of POU-, Hox-, bHLH-, zinc finger-, ETS- and Forkhead classes of transcription factors was applied in order to amplify members of these gene families from rat entorhinal cortex and cerebellum during the period of extensive axonal outgrowth, P0 and E18, respectively. Successful PCR amplifications were achieved in both brain regions tested with the POU and zinc finger primers. Employing degenerate POU primers, two class III family members, *brain-2 (brn-2)* and *brain-4 (brn-4)*, were amplified. The brain factors seem to be the predominant POU domain transcription factor family members expressed in brain at the time of neuronal differentiation as a high redundancy was obtained in the degenerate PCR with POU-primers. 35 analysed POU cDNAs yielded no more than two different POU factors. *Brn-2* and *brn-4* are strongly expressed and broadly distributed in the brain early on during development and continue to be expressed in adulthood (Alvarez-Bolado et al., 1995; He et al., 1989; Le Moine and Young, 1992; Mathis et al., 1992; Schreiber et al., 1993). In contrast, the type IV *brain* factors, including *brn-3a*, *brn-3b* and *brn-3c,* have a more distinct distribution in the CNS and appear to regulate the development of certain specific sensory functions (He et al., 1989; reviewed by McEvilly and Rosenfeld, 1999). As the expression pattern and function of *brn-2* and *brn-4* are already well studied during brain development and in the adult (Hagino-Yamagishi et al., 1997; He et al., 1989; Le Moine and Young, 1992; Malik et al., 1997; Mathis et al., 1992; reviewed by McEvilly and Rosenfeld, 1999; Nakai et al., 1995; Rosenfeld et al., 1996; Schonemann et al., 1995; Sharp and Morgan, 1996), no further examinations of these molecules were performed.

The identification of transcription factors of the zinc finger family contained some limitations although the amplification of zinc finger domain containing genes and generation of the characteristic “ladder” banding pattern were successful. As the region amplified by degenerate zinc finger primers was highly conserved and the sequence-span between the two primer binding sites quite short, the amplified PCR-fragments were elongated by 3’-RACE with the aim to obtain DNA regions outside the conserved domains which were specific for individual zinc finger factors. This should allow for reliable comparisons with sequences present in the databases and generation of sequence-specific RNA-probes for hybridisation. Yet, RACE is an intricate method that is useful for identification of the ends of a distinct cDNA-fragment but is not practical for elongation of a whole set of PCR-amplified zinc finger cDNAs. Accordingly, elongation of the 3’-end of amplified zinc finger cDNAs succeeded only in one case. Yet, hybridisation with this probe did not result in specific signals. However, the databases had been considerably enriched with additional information especially about mammalian sequences in the last years, which would render a future identification of zinc finger transcription factors by searching for overlapping sequences with highly conserved sequence stretches much easier. As the RNA probe generated for the one identified zinc finger cDNA did not result in specific hybridisation signals and the RACE-method did not seem to be effective in yielding multiple zinc finger sequences outside the conserved region, identification of zinc finger transcription factors was not further pursued.

Use of other degenerate oligonucleotides resulted in either no amplification products or in unspecific amplification of multiple cDNA fragments. This might be due to the fact, that degenerate primers were designed such that they were convenient for amplification of different subclass members of transcription factor families, which involves a higher degree of degeneracy. Hox genes, for example, all contain related homeodomain sequences, which can be classified into at least 30 distinguishable classes (Kappen et al., 1993). Sequences belonging to the same class are highly similar to each other but there are no obvious close relationships between the separate classes (Banerjee-Basu and Baxevanis, 2001; Kappen et al., 1993). The same is true for other transcription factor families, e.g. the ETS factors

(Janknecht and Nordheim, 1993; Wasylyk et al., 1993), bHLH proteins (reviewed by Bertrand et al., 2002; Massari and Murre, 2000) and Forkhead proteins (reviewed by Carlsson and Mahlapuu, 2002; Kaestner et al., 2000). With the intention to achieve a broad binding of the degenerate primers to members of different subclasses within one transcription factor family, degeneracy of the designed oligonucleotides might have become too high. This would explain the low yield that was obtained in the degenerate PCR using oligonucleotides for bHLH, HOX, ETS and FKH genes. Conversely, the POU-primers used in the described approach were specific only for one subclass of POU proteins (class III), leading to the sole amplification of class III-proteins and thereby confining the yield markedly. One strategy to circumvent this problem in the future would be to use sets of degenerate primers in the PCR for one transcription factor family, varying in one or two nucleotide triplets and thus amplifying the conserved domain of factors belonging to diverse subclasses. In addition, efficiency of degenerate PCR might also be increased by using primers tagged by unrelated sequences, e.g. derived from RNA-polymerase promotors, which can be bound by a second primer pair, e.g. promotor primers. This allows a higher annealing temperature in the second PCR thereby leading to a reduction of background and enhancement of specificity as demonstrated in a PCR with degenerate zinc finger primers (Agata et al., 1998).

To conclude, the paradigm of the degenerate PCR aimed at the identification of transcription factors, which were highly expressed in the rat cerebellum and entorhinal cortex, respectively, at the time of substantial axonal outgrowth. Further interest lied in the spatial and temporal distribution of identified transcription factors in the developing brain, which implicated subsequent expression analyses of the PCR-amplified cDNAs by Northern- and in situ-hybridisation experiments. Although the degenerate PCR approach was proficient for amplification of conserved regions of POU-and zinc finger transcription factors, it was not efficient in producing various template sequences which could be used for the generation of specific probes for subsequent expression studies of each amplified transcription factor.

4.2 Suppression subtractive hybridisation (SSH)

The method of SSH was applied with the aim of identifying differentially expressed genes, which are highly expressed in the postnatal rat brain during the period of extensive axonal outgrowth and whose expression decreases with progression of development. By this means, it seemed very promising to find genes that might play a role in neuronal differentiation processes during postnatal development and might possibly be involved in axon growth. Indeed, a considerable part of our subtracted, known genes had previously been examined and was shown to be downregulated during brain development (indicated by an asterisk in *tables 3-1 and 3-3* in sections 3.2.1.and 3.2.2, respectively). mRNA expression of p19/stathmin, for example, has been extensively studied in the rat and a greater abundance in newborn than in adult brain has been demonstrated (Sugiura and Mori, 1995). Thymosin β_4 shows high expression during granule cell migration and parallel fiber elongation in rat cerebellum early in postnatal development but expression declines thereafter (Border et al., 1993; Lin and Morrison-Bogorad, 1990). The neuronal surface glycoprotein OX2 as well is differentially regulated and displays highest protein levels during axogenesis in rat cerebellum (Morris and Beech, 1987). Developmentally regulated molecules were also present in the entorhinal subtraction, e.g. GAP-43, which is one of the best characterized proteins that is associated with axon growth and regeneration, and whose expression correlates well with process outgrowth during development and lesion-induced sprouting (Aigner et al., 1995; Buffo et al., 1997; Caroni, 1997; Laux et al., 2000; reviewed by Skene, 1989; Skene and Willard, 1981b). Another molecule, Bdm2, is more abundantly expressed in fetal than in adult brain (Nishinaka et al., 2000). Though few of the subtracted developmental molecules represent early markers like nestin, which is strongly expressed in stem cells or proliferating cells, numerous genes are upregulated later during development, correlating well with the period of extensive axon growth, and some of them were shown to be associated with axonal regeneration. For instance, SCG-10 mRNA expression is localized within growing neurites, peaks during neurite outgrowth and is associated with axon regeneration (Hannan et al., 1996; Mason et al., 2002; Pellier-Monnin et al., 2001). HB-GAM expression is high in developing axonal projections (Rauvala et al., 1994) and stathmin is developmentally regulated during axon growth in the rat and is transiently induced after EC-lesion (Brauer et al., 2001).

These results confirm, that the applied strategy was very efficient for enrichment of differentially regulated genes that are expressed more abundantly in developing than in mature CNS. The two subtracted libraries thus provide valuable tools to identify candidate molecules that are strongly expressed in rat brain around birth and might play a role in axonal outgrowth. In the following, the temporal and spatial expression pattern and possible functional significance of those identified molecules, which were studied in more detail, will be discussed.

4.3 CD24

One of the identified genes in the cerebellar subtraction approach was the cell adhesion molecule CD24, which is developmentally regulated in brain and can either stimulate or inhibit axon outgrowth, depending on the cell type and on other cell adhesion molecules expressed by these cells (Kleene et al., 2001; Kuchler et al., 1989; Nedelec et al., 1992; Shewan et al., 1996; Shirasawa et al., 1993). Rat CD24 is a highly glycosylated protein of

31 kDa that is attached to the cell membrane via a glycosyl phosphatidylinositol (GPI) anchor and is, in addition to neuronal cells, expressed by cells of the hematopoietic lineage, keratinocytes, epithelial cells and carcinoma cells (Jackson et al., 1992; Kay et al., 1991; Kay et al., 1990; Magnaldo and Barrandon, 1996; Shirasawa et al., 1993; Wenger et al., 1991). It is widely expressed in the nervous system during embryonic development and displays strongest expression in embryonic brain, spinal cord, dorsal root ganglia, retina and on optic nerve fibers (Shewan et al., 1996; Shirasawa et al., 1993). In the adult brain, CD24 is dramatically downregulated (Nedelec et al., 1992; Poncet et al., 1996; Shirasawa et al., 1993) and expression persists only in regions of secondary neurogenesis, the subventricular zone of the lateral ventricle and the dentate gyrus (Calaora et al., 1996).

In the present study, CD24 expression was examined in various brain regions in the late embryonic and early postnatal development of the rat by Northern blotting and in situ-hybridisation. Northern analysis revealed a strong decline of CD24 transcript in cerebral cortex, hippocampus and brainstem between E18 and P10. This is in accordance with previous studies in the mouse, which demonstrated that CD24 protein levels decrease in a similar time course in the forebrain (Calaora et al., 1996; Nedelec et al., 1992). Moreover, in the embryonic rat, CD24 was shown to be exclusively expressed in postmitotic neurons of the neuroepithelium, which were devoid of BrdU incorporation that selectively labels proliferating cells (Shirasawa et al., 1993).

The present Northern analysis further revealed CD24 transcript levels in the cerebellum until P10 with strongest expression at P0, but no detection was found at P35. In situ-hybridisation confirmed this temporal regulation and demonstrated that, whereas CD24 mRNA was strongly expressed in the Purkinje cell layer and deep cerebellar nuclei at E20, expression decreased markedly during early postnatal development, became restricted to the external granular layer and further diminished with increasing age. This does not entirely match previous immunhistochemical studies (Calaora et al., 1996), in which was noted that CD24 protein expression in the mouse cerebellum was low before birth, increased between P0 and P6, declined thereafter and had disappeared around P10. Kuchler et al. observed a transient expression of CD24 protein in postmitotic, pre-migratory granule cell precursors in the internal part of the external granular layer, on parallel fibers and in the white matter of the developing cerebellum in rat and mice, but did not detect expression in Purkinje cells (Kuchler et al., 1989). This might be due to the later time point of their developmental studies, starting at P5, when CD24 transcript levels in Purkinje cells are already substantially decreased. CD24 might be expressed by Purkinje neurons around birth and transported into the axonal compartment where it might be anchored to the cell membrane. This would explain the intense immunostaining of axonal tracts in the cerebellar white matter and the lack of labelling of Purkinje cell bodies described by

Kuchler et al. between P5 and P15 (Kuchler et al., 1989). Moreover, the faint staining of Purkinje cells in the adult (see *fig. 3-19*, J and K) is consistent with the finding by Kuchler et al. that the cerebellar white matter is still weakly positive for CD24 protein in the adult mouse (Kuchler et al., 1989). The strong transcription of CD24 by deep cerebellar neurons and cells at the isthmal region of the cerebellum detected at E20 (see *fig. 3-19*, A and B) could contribute to the intense immunostaining found on axons in the cerebellar white matter by Kuchler et al, too.

Although the precise function of CD24 in the nervous system has not yet been determined, its transient expression by postmitotic neurons and on developing axon tracts during neuronal migration and axonal elongation suggests that it might play a role in these processes during development (Calaora et al., 1996). This was confirmed by experiments with dissociated, cultured neurons, in which CD24 was demonstrated to exert differential effects on neurite outgrowth, depending on the neuron type and on the developmental age (Kleene et al., 2001; Shewan et al., 1996). Interestingly, Kuchler et al. found that CD24 decreased in the cerebellar white matter during myelination. In addition, CD24-deficient mice revealed enhanced proliferation in regions of secondary neurogenesis, which suggests a role for CD24 in negatively regulating cell proliferation in these zones by either maintaining precursor cells in a quiescent state or initiating differentiation (Belvindrah et al., 2002). Heterophilic interaction was proposed as the mode of action of CD24 since GPI-anchored CD24 lacks an intracellular domain and therefore itself cannot transduce signals from the extracellular milieu to the intracellular compartment. CD24 was shown to interact with another cell recognition molecule in neurons, the glycoprotein L1, thereby activating the signal transduction pathway of L1 (Kadmon et al., 1995; Kleene et al., 2001). Moreover, it was found that glycan chains and sialylation on the CD24-core peptide play an essential role for exertion of the differential effects of this molecule on neurite outgrowth (Kleene et al., 2001).

The present results clearly show that CD24 is massively transcribed in the rat cerebellum before birth and that Purkinje neurons, deep cerebellar neurons and neurons from an extracerebellar nucleus are the main regions of synthesis. Moreover, it is illustrated that CD24 transcripts are significantly downregulated in these neurons between E20 and P4, when their axonal connections are established.

4.4 rCRHSP-24

Suppression subtractive hybridisation further identified the transcript for rCRHSP-24, a protein which had not previously been associated with a differential expression in the developing rat brain during the period of extensive fiber outgrowth. rCRHSP-24 was known in the rat before and its mRNA was previously identified (Groblewski et al., 1998).

Yet, the identified cDNA fragment for CRHSP-24 first did not match known mRNAs in the sequence databases, as it constituted the 3' -untranslated region adjacent to the transcript's polyA tail whereas the known rat CRHSP mRNA in the database comprised only the coding region of this transcript. This demonstrates that the inability to find sequence database matches for some of the subtracted clones (29 cases in total for both subtraction approaches) could be a consequence of targeting the polyA tail of mRNAs with the suppression subtractive hybridisation approach. These clones, as turned out for CRHSP-24, might represent the untranslated regions of a characterized mRNA whose sequence in this region is unknown. Concerning CRHSP-24, the identified clone was found to be the 3'-untranslated region of the rat transcript because of its strong homology to the full-length mRNA in the mouse. This emphasizes that for those subtracted clones, which did not match any known sequences in the databases, screening in the EST-divisions of the databases for overlapping rat cDNA sequences and use of these in subsequent searches might be helpful to identify additional known mRNAs among the subtracted clones.

In Northern analysis, two CRHSP-24 transcripts of 2,9 and 1,7 kilobases were detected in various rat brain tissues from late embryonic development until adulthood. In previous studies, Groblewski et al. examined CRHSP transcript expression in multiple adult rat tissues, including adult brain, and noted that in all tissues tested, two transcripts of 2,9 kb and 0,7 kb, respectively, were expressed (Groblewski et al., 1998). The 2,9 kb-transcript most probably corresponds to the full length CRHSP-24 mRNA in rat since a full length mRNA for CRHSP-24, which is similar in size (2847 bp) to the 2,9 kb transcript, is identified in the mouse (accession number: AK004711). Whereas the 2,9 kb transcript detected in the present Northern analysis is consistent with the results from Groblewski et al., detection of an additional 1,7 kb transcript is not. It can be supposed that both different transcripts arise by alternative splicing as a search in the databases of rat genomic sequences revealed the existence of two intron sequences in the coding parts of the rat CRHSP-24 gene thus leading to the generation of two distinct proteins. Yet, Groblewski et al. demonstrated only a single 24-kDa protein in multiple tissues including brain by immunoblotting with a polyclonal antiserum raised against full-length CRHSP-24 protein. It is thus likely that the protein generated from the smaller transcript might differ in its epitopes from the full-length protein, thus preventing it from recognition by the polyclonal antiserum used by Groblewski et al. Alternatively, the smaller transcript might also be unstable or posttranscriptionally regulated and translation of this mRNA might be repressed. However, this does not explain the discrepancy between the two different transcript sizes (0,7 kb and 1,7 kb) for the smaller mRNA species detected by Groblewski et al. and in the present studies.

Expression analysis of CRHSP-24 by Northern hybridisation at different developmental stages revealed that the larger of the two transcripts is clearly downregulated during postnatal development and is only weakly expressed in adult brain tissues. Although Groblewski et al. clearly detected the 2,9 kb CRHSP-24 transcript in the adult brain, expression levels were markedly weaker compared to levels in other tissues such as liver or lung and quantity of RNA blotted was much higher.

rCRHSP-24, initially isolated from rat pancreas, is a calcium-regulated phosphoprotein and a substrate for the protein phosphatase calcineurin (Groblewski et al., 1998). It contains a conserved cold shock DNA/RNA-binding domain that was originally discovered in bacterial cold shock proteins (Groblewski et al., 1998). Prokaryotic and eukaryotic proteins containing such domains are believed to function as RNA chaperones in prokaryotes and as transcriptional and translational regulators in eukaryotes (Jones and Inouye, 1994). CRHSP-24 was proposed to modulate expression of stage-specific mRNAs in mouse testis during differentiation of spermatids into spermatozoa by complexing with the Styx /dead phosphatase protein (Wishart and Dixon, 2002). CRHSP-24 hence might be a calcium-stimulated regulator of transcriptional or translational gene expression. As CRHSP-24 transcript is highly expressed in brain of embryonic and newborn rats and is strongly downregulated during early postnatal development in cortex and hippocampus and later in development in cerebellum and brainstem, it might be implicated in Ca^{2+}/calcineurin-mediated signal transduction in the developing brain and might exert repression or stimulation of gene expression during neuronal differentiation. Since the calcium/calmodulin-regulated protein phosphatase calcineurin, which dephosphorylates CRHSP-24 following a rise in free cellular calcium (Groblewski et al., 1998), is expressed in many developing rat brain regions not until P4 (Polli et al., 1991), other mechanisms are likely to regulate activation of CRHSP-24, too. This is consistent with the finding of Groblewski et al. that CRHSP-24 is constitutively dephosphorylated by a phosphatase other than calcineurin in pancreatic acinar cells (Groblewski et al., 1998). It therefore would be an interesting task to identify binding partners of CRHSP-24 in the brain, examine its intracellular localization and elucidate its function in neuronal development.

4.5 rMMS2

Another molecule identified by the cerebellar subtraction approach was rMMS2. rMMS2 is a novel rat gene that was first characterized in the rat in this study, and which was first shown to be differentially regulated during postnatal brain development. Northern hybridisation revealed a major 1,7 kb rMMS2 transcript in rat cerebral cortex, hippocampus, cerebellum and brainstem and a minor transcript, 2,7-3,7 kb in size, which, however, was only observed in cerebral cortex and hippocampus at E18 with the cRNA probe concentrations used in the present studies. Several transcripts were also demonstrated in adult mouse brain, with a major band at 1,4 kb (Franko et al., 2001), whereas a single transcript (1,5 kb) was observed in human tissues (Xiao et al., 1998). The 1,7 kb transcript, which seems to be the major transcript expressed in the rat CNS, is likely to be generated by alternative splicing as the rat gene for rMMS2 consists of several exons. Northern analysis at different developmental stages demonstrated a strong downregulation of the 1,7 kb rMMS2 mRNA during postnatal development and neuronal differentiation with only weak expression in adult brain. This is in line with the human homologue, which was hardly detectable in adult brain (Xiao et al., 1998), but is in contrast to the mouse MMS2 transcript that was reported to be strongly expressed in adult brain (Franko et al., 2001).

rMMS2 belongs to the family of ubiquitin-conjugating enzyme variants (UEVs), which are highly conserved proteins (Villalobo et al., 2002) that share structural similarity with ubiquitin-conjugating E2 enzymes (Ubcs) but lack the critical cysteine residue essential for the catalytic activity and, in vitro, do not transfer ubiquitin to protein substrates in the second step of ubiquitination (Franko et al., 2001). Yeast MMS2 was the first UEV that had been identified (Broomfield et al., 1998). It plays a role in error-free DNA postreplication repair to limit spontaneous and damage-induced mutagenesis and is transcriptionally upregulated in response to DNA damage (Broomfield et al., 1998; Ulrich, 2001). Mouse and human MMS2 proteins are able to complement the mms2 null mutant in yeast and therefore seem to act in the same postreplication repair pathway as the yeast counterpart (Franko et al., 2001; Xiao et al., 1998). MMS2 of yeast, mouse and human were shown to associate with the E2 enzyme Ubc13, which results in assembly of novel polyubiquitin-chains for DNA-repair (Ashley et al., 2002; Hofmann and Pickart, 1999; McKenna et al., 2001; Torres-Ramos et al., 2002). Yeast MMS2 and to a lower level also human MMS2 are capable of transcriptionally activating the promotor for the c-fos proto-oncogene (Xiao et al., 1998), whose expression is necessary for protection of cells against DNA damaging agents (Kaina et al., 1997). Human MMS2 was also isolated as enterocyte differentiation promoting factor EDPF-1 (EMBL/GenBank/DDBJ databases, Accession number: U62136, Faria J, 1999), but the function of MMS2 during differentiation was not examined. In summary, MMS2 plays a role in DNA repair and is possibly involved in ubiquitination. It might exert these functions by formation of heterodimers with Ubcs or E3 ubiquitin ligases, thereby modifying their intracellular localization, substrate specifity and activity. This could be a possible strategy of eukaryotic cells to increase diversity and selectivity in ubiquitin conjugation (Sancho et al., 1998).

Evidence exists that ubiquitination is also important for the assembly and function of neuronal circuits and that it is indeed critical to the growth cone behaviour at the midline in

Drosophila (Murphey and Godenschwege, 2002). *Comm*, the *Robo*-adaptor protein *commissureless*, regulates *Robo* removal from the growth cone surface as the growth cone approaches the midline. Recent studies suggest that ubiquitination of *Comm* and/or *Robo* plays a role in regulating *Robo* removal by affecting protein trafficking (Georgiou and Tear, 2002; Keleman et al., 2002; Myat et al., 2002). The first evidence that the ubiquitin pathway is involved in axon growth and guidance came by identification of an ubiquitin-conjugating enzyme in Drosophila, which, when mutated in the catalytic domain, leads to pathfinding errors near the target cells in several neuronal projections (Muralidhar and Thomas, 1993; Oh et al., 1994). Moreover, *in vitro* experiments from Campbell et al. point to an important function for the ubiquitin system in growth cone attraction by Netrin/DCC signalling (Campbell and Holt, 2001). In addition to these functions in axon pathfinding and growth cone decisions at choice points, ubiquitin seems also to be involved in the establishment of neuromuscular synapses by removal of *Comm* from the muscle surface (Wolf et al., 1998). Synaptic stabilization during development and synaptic plasticity are regulated by ubiquitin as well (Burbea et al., 2002; DiAntonio et al., 2001; Fischer and Overstreet, 2002; reviewed by Hegde and DiAntonio, 2002; Hicke, 1999). Altogether, ubiquitination seems to be well implicated in the molecular remodelling and dynamic regulation of protein trafficking that occurs in growth cones and synapses.

Furthermore, new findings suggest, that ubiquitination also plays a direct role in activation or suppression of gene expression (Conaway et al., 2002; reviewed by Hegde and DiAntonio, 2002; Ostendorff et al., 2002; Salghetti et al., 2001). Whether UEVs like MMS2 are part of such signalling pathways (possibly as regulators of E2 and E3 enzymes) is unknown and their functional role remains to be investigated. Interestingly, the highest expression of rMMS2 in rat brain is around birth in postmitotic neurons during the period of fiber outgrowth. This temporal regulation of expression suggests a distinct or additional role to DNA repair for rMMS2 in rat brain during neuronal differentiation.

4.6 Conclusions and summary of the suppression subtractive hybridisation

In order to identify molecules that might positively influence axon growth, the subtraction approaches with cerebellum and entorhinal cortex at two developmental stages were performed in such a way that it was searched for positively acting, growth-promoting molecules, which are more abundant at the younger stage than in the more mature population. Yet, it is clear that with a subtraction approach, in which transcripts from mature, growth-incapable neurons become subtracted from young, growth-capable neurons, only genes can be identified that exhibit a pronounced downregulation during neuronal maturation. Of course, growth-associated genes might also be expressed continuously throughout postnatal neuronal development, in which case their growth-promoting function might be masked or inhibited by negatively acting molecules with progression of development. Myelin-associated growth inhibitors, whose expression arises during maturation of CNS neurons at the period when the growth-potential sharply declines (reviewed by Kapfhammer, 1997), might function as such negative regulators. They might influence the expression or signal transduction of growth-promoting molecules by retrograde regulation. It was shown, for example, that the lesion-induced cell body response is enhanced in Purkinje neurons after neutralization of myelin-associated growth inhibitors (Zagrebelsky et al., 1998). Moreover, target-derived retrogradely transported repressive factors might account for the decline of growth-associated proteins such as GAP-43 during neuronal maturation (Karimi-Abdolrezaee and Schreyer, 2002). A subtraction approach performed vice versa (subtract young from old), thus identifying sequences that are exclusively expressed in the mature neuronal population, would consider such inhibitory genes, which might become upregulated in the course of neuronal differentiation and which might negatively regulate growth-promoting genes as neuronal maturation proceeds.

To summarize, the gradual decline in the axon growth capability of maturing CNS neurons may be attributable to either intrinsic modifications associated with the acquisition of the adult neuronal phenotype (Bates and Meyer, 1997), the progressive appearance of growth inhibitory signals (reviewed by Kapfhammer, 1997) or disappearance of growth-promoting factors (Kalil and Skene, 1986; reviewed by Skene, 1989). Concerning the first issue, an intrinsic change of gene expression patterns of CNS neurons during postnatal development and differentiation, the results presented here are a fist step towards identifying molecules that might be associated with axon growth. By the method of suppressive subtractive hybridisation, two subtractive libraries from E18-cerebellum and P0-entorhinal cortex, respectively, were generated, which allowed the enrichment of genes that are actively transcribed in rat brain around birth but which are silent or much less transcribed after establishment of neuronal circuits. Several genes were identified that exhibited a distinct temporal regulation during postnatal brain development with higher expression levels during the young, immature developmental stage. The efficiency of this approach was proven by the enrichment of numerous genes whose developmental expression or association with axon growth had previously been described. Two molecules, rCRHSP-24 and the novel rat gene rMMS2, were demonstrated for the first time to be highly expressed in neurons of various brain regions at the neonatal stage and to be extensively downregulated after the critical period of axon elongation. Although the precise role of both proteins during neuronal development is not yet clear, rMMS2, as possible player in ubiquitin-mediated modification of target proteins, and rCRHSP-24, which probably is implicated in Ca^{2+}/calmodulin-mediated signalling pathways, might function as indirect modulators of axon growth. At least, their high expression during axonal outgrowth in

CNS neurons in many brain regions and their strong downregulation during postnatal development point to a role of these proteins in neuronal differentiation of CNS neurons.

4.7 Outlook

As two molecules, rMMS2 and CRHSP-24, whose expression or function in nervous system development had not been examined before, exhibited a strong downregulation in CNS neurons during postnatal maturation, it would be of great interest to follow up these subtracted candidates. As a first step, the expression of MMS2 and CRHSP-24 proteins should be analysed in order to find out whether the regulation of these two molecules occurs similarly on the transcript and protein level. If so, it is obvious that future experiments would concentrate on the function that both proteins occupy during differentiation of CNS neurons. One way to study this could be in slice cultures as organotypic cultures with cerebellum and entorhinal cortex, respectively, are well established (Dusart et al., 1997; Li et al., 1995; Prang et al., 2001; Woodhams et al., 1993). Applying the method of antisense-oligonucleotides on cerebellar slices or entorhino-hippocampal co-cultures at the time when both genes normally are highly expressed, one could investigate the effects of blocking the transcription of rMMS2 and CRHSP-24, respectively. Since transcripts of rMMS2 and CRHSP-24 are very abundant in CNS neurons during the period of axonal outgrowth and axonal projections develop well in the two slice culture models, it could be nicely studied whether blocking the subtracted candidate molecules would have any effect on axon growth of Purkinje neurons and entorhinal pyramidal cells, respectively. Conversely, one could try to force the expression of rMMS2 and CRHSP-24 in these neurons, e.g. by transfection with a gene gun or viral infection, at a time point when expression of both molecules is already downregulated. As this later developmental stage correlates with the strong decline in axon growth-potential of CNS neurons, it would be attractive to investigate whether ectopic expression of both proteins could ameliorate the growth behaviour of mature Purkinje cells and entorhinal pyramidal neurons. Moreover, co-immunprecipitation experiments or the yeast two-hybrid system could be applied in order to identify possible interaction partners of rMMS2 and CRHSP-24. This would be a first step towards elucidating the signal cascades involved in mediating rMMS2 and CRHSP-24 actions. Immunfluorescence analysis could provide information about the intracellular localisation of the proteins, which would be of interest in regard to the possible functions of both proteins in DNA-binding that occurs in the nucleus.

Furthermore, many other subtracted clones are still awaiting their further analysis. Expression studies of yet uncharacterised subtracted cDNAs could yield additional interesting candidate molecules that are downregulated during postnatal development.

5 Summary

During neuronal development CNS neurons extend axons over long distances. This high growth potential is lost during postnatal development resulting in very poor axonal outgrowth and regeneration in the adult CNS. This pronounced decline of axon growth potential and regenerative capability might be related to alterations in the expression level of growth-associated genes during postnatal development. The aim of the present study was the identification of candidate molecules that might be associated with axon growth, i.e. which are strongly expressed during axonal outgrowth and are downregulated as neuronal maturation proceeds. As the time periods of developmental axonal outgrowth and decrease in growth potential are well studied in rat cerebellum and entorhinal cortex, these two brain regions were chosen as model systems for analysis of gene expression patterns during axonal extension and after completion of pathway formation. In a first approach the study focused on the identification of transcription factors, because they are known to be involved in the regulation of cellular identity and differentiation and hence might also determine the intrinsic growth state of a neuron. In order to identify transcription factors from rat cerebellum and entorhinal cortex at the time of maximal axonal outgrowth, PCR with degenerate oligonucleotides, specific for the conserved DNA-binding domains of distinct transcription factor classes, was performed with cDNA from cerebellum at E18 and entorhinal cortex at P0, respectively. A limited number of PCR products could be isolated from the above brain regions by the use of primers for the POU and zinc finger family of transcription factors. Because of the small number of candidate molecules and considerable difficulties in constructing cDNA probes for further analysis this approach was not further pursued. A second approach aimed at the comparison of the transcriptional activity of young differentiating CNS neurons, which extend axons, with that of more mature neurons, which have lost growth competence. The method of suppression subtractive hybridisation (SSH) was performed in two distinct CNS tissues, rat cerebellum and entorhinal cortex, at two developmental stages (E18 and P35 for cerebellum and P0 an P10 for entorhinal cortex, respectively) in order to enrich for genes, which are downregulated during postnatal development. Several differentially expressed genes were identified, and the temporal and spatial expression pattern of some of these genes was further examined in rat brain by Northern- and in situ-hybridisation analysis at different developmental stages. One of the identified genes, rMMS2, was not known in the rat before and was characterised in this study for the first time. In addition, CRHSP-24, whose expression pattern had not previously been examined in the developing brain, was identified as a differentially expressed gene. Further analysis showed that rMMS2 and CRHSP-24 were strongly expressed in many brain regions during late embryonic and early postnatal development. Expression of both genes was significantly downregulated during the first postnatal weeks and was only weak or absent in the adult brain. As this regulated distribution correlates well with the time period of establishment of axonal connections in the developing brain, these molecules might play a role in neuronal differentiation processes. However, their function in neuronal development is not yet clear and remains to be elucidated. Because only a fraction of the enriched genes has been analysed by now the pool of subtracted genes might serve as a valuable source for the identification of further candidate genes, which might be associated with neuronal differentiation and axonal outgrowth.

6 Zusammenfassung

Nervenzellen (Neurone) des Zentralen Nervensystems (ZNS) bilden während der Entwicklung des Nervensystems lange Fortsätze aus, die Axone, die in das zukünftige Innervationsgebiet wachsen, um dort synaptische Kontakte mit den Zielneuronen zu formen. Dieses Auswachsen der Axone ist auf eine relativ kurze Zeitspanne während der Entwicklung des Gehirns begrenzt. Ist diese Periode verstrichen, geht die Fähigkeit der Neurone zum Axonwachstum stark zurück. Dies führt unter anderem dazu, dass im adulten Gehirn nach Durchtrennung bestehender Axone kaum axonale Regeneration möglich ist. Ein wichtiger Grund für das mangelhafte Faserwachstum in adulten, ausdifferenzierten Neuronen könnte das Fehlen von Molekülen sein, die das Axonwachstum anregen oder positiv beeinflussen, und die in Neuronen des ZNS nur während der Periode des massiven Axonwachstums in der Entwicklung, aber nicht mehr zu einem späteren Zeitpunkt gebildet werden. Das Ziel der vorliegenden Studien war deshalb, solche Moleküle zu identifizieren, die während der Periode des Axonwachstums in Neuronen des ZNS stark exprimiert werden, deren Expression aber zu einem späteren Zeitpunkt, wenn die axonalen Verbindungen fertig ausgebildet sind, erheblich zurückgeht. Da das kritische Zeitfenster, in dem Faserwachstum möglich ist, im Cerebellum und Entorhinalen Cortex der Ratte zuvor genau untersucht und beschrieben worden war, eigneten sich diese beiden Gehirnregionen besonders gut, um Axonwachstums-assoziierte Kandidatenmoleküle zu suchen. Zwei Methoden wurden hierfür angewandt. Die erste, die Methode der „degenerierten PCR“, fokussierte auf eine bestimmte Molekülklasse, die Transkriptionsfaktoren, da diese regulatorisch wirksame Proteine sind, welche die Expression vieler Zielgene beeinflussen und eine wichtige Rolle bei der Entwicklung des Nervensystems spielen. Mittels PCR mit degenerierten Primern wurden die konservierten DNA-Bindungsdomänen verschiedener Klassen von Transkriptionsfaktoren, zum Beispiel der POU- oder Zinkfinger- Familie, amplifiziert. Wegen der geringen Zahl an isolierten Molekülen und Schwierigkeiten bei der Herstellung von spezifischen RNA-Sonden für eine anschließende Hybridisierung wurde dieser Ansatz nicht weitergeführt. Deshalb wurde eine zweite Methode, die „Subtraktive Hybridisierung“, angewandt. Diese Methode erlaubt es, zwei verschiedene mRNA-Populationen miteinander zu vergleichen und solche Transkripte zu finden, die ausschließlich oder verstärkt in einer der beiden Gruppen vorhanden sind. Hier wurden die mRNA-Populationen aus zwei verschiedenen Entwicklungsstadien miteinander verglichen, einem frühen Stadium, wenn Axone auswachsen, und einem späteren, wenn kein Axonwachstum mehr möglich ist. Diese vergleichenden Studien wurden in zwei unterschiedlichen Ansätzen einmal im Cerebellum der Ratte und zum anderen im Entorhinalen Cortex der Ratte durchgeführt. Damit konnten mehrere Gene identifiziert werden, die differenziell reguliert werden, das heißt, die in jungen, auswachsenden

Neuronen stark exprimiert werden, die jedoch nach der kritischen Periode herunterreguliert werden und in adulten Neuronen kaum mehr vorhanden sind. Eines der subtrahierten Kandidatengene, rMMS2, war in der Ratte noch unbekannt und wurde hier zum ersten Mal beschrieben. Dieses und ein weiteres Gen, CRHSP-24, dessen Aktivität im sich entwickelnden Gehirn der Ratte noch nicht beschrieben war, wurden mittels Northern- und in situ-Hybridisierungen auf ihre Expression hin untersucht. Es konnte gezeigt werden, dass beide Gene zum Zeitpunkt der Geburt in vielen Gebieten des Gehirns stark exprimiert werden, ihre Expression aber während der postnatalen Entwicklung erheblich zurückgeht und im adulten Gehirn nur noch schwach nachgewiesen werden kann. Dieser Zeitverlauf entspricht der Entwicklungsperiode, in der viele axonale Verbindungen gebildet werden und lässt darauf schließen, dass diese Proteine bei neuronalen Differenzierungsprozessen eine wichtige Rolle spielen könnten. Ihre genauere Funktion ist allerdings noch ungeklärt und bedarf weiterer Untersuchungen. Bisher konnte nur ein kleiner Teil der durch die subtraktive Hybridisierung identifizierten Gene genauer untersucht werden. Alle übrigen Gene, die durch diese Methode angereichert und bislang noch nicht charakterisiert wurden, bilden einen interessanten Ausgangspunkt für die Identifizierung weiterer Kandidatengene, die an der Regulation von neuronaler Differenzierung und axonalem Wachstums beteiligt sein könnten.

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8 Appendix

8.1 Supplier list

Apelex	Z.I. de la Bonde, 6, rue Marcel Paul, 91742 Massy Cedex, F
AppliChem GmbH	Ottoweg 10b, 64291 Darmstadt, D
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Bio-Rad Laboratories AG	Nenzlingerweg 2, 4153 Reinach BL, CH
Catalys AG (Promega)	Industriestrasse 28, 8304 Wallisellen, CH
Charles River Lab., Inc.	251 Ballardvale Street, Willmington, MA 01887-1000, USA
Clontech Laboratories AG	Matthäusstrasse 18, 4057 Basel, CH
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Fluka Chemie GmbH	Industriestrasse 25, 9471 Buchs, CH
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Heraeus	see: Kendro
IKA-Werke GmbH & Co. KG	Jahnke & Kunkel-Strasse 10, 79219 Staufen, D
Intas GmbH	Florenz-Sartorius-Strasse 14, 37079 Göttingen, D
Invitrogen AG	Uferstrasse 90, 40190 Basel, CH
R. Jung GmbH	Heidelberger Strasse 17-19, 69226 Nussloch, D
Kendro Laboratory Products AG	Räffelstrasse 32, Postfach, 8045 Zürich, CH
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Merck Sharp & Dohme-Chibret AG	Schaffhauserstrasse 136, 8152 Glattbrugg, CH
Mettler-Toledo AG	Im Langacher, P.O. BOX, 8606 Greifensee, CH
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Pharmacia AG (Amersham Biosciences Europe)	Lagerstrasse 14, 8600 Dübendorf, CH
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Qiagen AG	Auf dem Wolf 39, 4052 Basel, CH
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Roth AG	Christoph Merian-Ring 7, 4153 Reinach BL1, CH
Schleicher & Schuell GmbH	Grimsehlstrasse 23, 37574 Einbeck, D
Sigma-Aldrich Chemie GmbH	Eschenstrasse 5, 82024 Taufkirchen, D
Stratagene Europe	P.O. Box 12085, 1100 AB Amsterdam, NL
Vaudaux-Eppendorf AG	Im Kirschgarten 30, 4124 Schönenbuch / Basel, CH

8.2 Abbreviation index

approx.	approximately
BCIP	5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt
BDNF	brain derived neurotrophic growth factor
bHLH	basic helix-loop-helix
bp	base pairs
CA1-CA3	cornu ammonis regions of the hippocampal formation
CAM	cell adhesion molecule
cAMP	cyclic adenosine monophosphate
cDNA	complementary DNA
CDR	coding region
CDS	coding sequence
CE	cerebellum
C.elegans	Caenorhabditis elegans
CNS	central nervous system
ddH$_2$O	distilled deionised water
DIG	digoxigenin
DMF	dimethylformamide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DRG	dorsal root ganglion
EC	entorhinal cortex
EDTA	ethylenediaminetetraacetate
E	embryonic day
E. coli	Escherichia coli
EST	expressed sequence tag
GDNF	glial derived neurotrophic growth factor
GFP	green fluorescent protein
HOX	homeobox

IEG	immediate early gene
Ig	immunglobulin
kb	kilo bases
kDa	kilo Dalton
LTD	long term depression
LTP	long term potentiation
M	molar
mM	milli molar
µg	microgram
MOPS	3-morpholinopropanesulfonic acid
mRNA	messenger ribonucleic acid
NBT	nitroblue tetrazolium chloride
NGF	nerve growth factor
NT	neurotrophin
ORF	open reading frame
P	postnatal day
PBS	phosphate buffered saline
PC12	phaeochromocytoma cells (a neuroendocrine cell line)
PCR	polymerase chain reaction
PNS	peripheral nervous system
RGC	retinal ganglion cell
RNA	ribonucleic acid
RNase	ribonuclease
SDS	sodium dodecyl sulfate
SSC	sodium citrate buffer
SSH	suppression subtractive hybridisation
UTR	untranslated region
UV	ultraviolet

8.3 Publications

Parts of this work were published as poster presentations or in scientific journals:

Hofsaess, U. and Kapfhammer J.P. **(2003)** Identification of numerous genes differentially expressed in rat brain during postnatal development and expression analysis of the novel rat gene rMMS2. *Brain Res Mol Brain Res,* in press

Hofsäss,U. and Kapfhammer J.P. Identification of genes differentially expressed in rat cerebellum and entorhinal cortex during development. *FENS Abstr.* vol 1, A138.10, **2002**

Hofsaess, U. and Kapfhammer J.P., Identification of genes differentially expressed in rat cerebellum and entorhinal cortex during development. *Soc. Neurosci. Abstr.*, Vol. 27, 795.8, **2001**

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Curriculum vitae

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Postgraduate studies

Oct. 1998-March 2003	PhD at the Institute for Anatomy in Basel, Switzerland and Freiburg, Germany: “Identification of genes differentially expressed in rat brain during postnatal development”; supervisor: Prof. Dr Josef Kapfhammer

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